UNIVERSITY OF HAW.
LIBRARY

THE JOURNAL

OF

Comparative Pathology

AND

Therapeutics

VOLUME LXVI

1956

EDITED BY

J. G. WRIGHT

T. J. BOSWORTH R. E. GLOVER E. G. WHITE

FOUNDED IN 1888

BY

JOHN M'FADYEAN

Reprinted with the permission of the original publishers

KRAUS REPRINT LTD.

VADUZ

1964

Printed in Germany

FUMIGATED 1995

57-34956 Lq
SF601
J68
v.66

INDEX TO AUTHORS IN VOLUME LXVI.

PAGE

ABDUSSALAM, M. (See Blakemore, F.)
ARTHUR, G. H. Data on Sheep Reproduction derived from Abattoir Material 345

BETTS, A. O., and CAMPBELL, R. C. The Action of Antibiotics and Sulphamezathine on the Causal Agent of Virus Pneumonia of Pigs 89
BLAKEMORE, F., and GARNER, R. J. The Maternal Transference of Antibodies in the Bovine 287
BLAKEMORE, F., and ABDUSSALAM, M. Morphology of the Elementary Bodies and Cell Inclusions in Swine Pox .. 373
BLAXLAND, J. D., SOJKA, W. J., and SMITHER, A. M. A Study of *Salm. pullorum* and *Salm. gallinarum* Strains isolated from Field Outbreaks of the Disease 270

CAMPBELL, R. C. (See Betts, A. O.)
CHODNIK, K. S. Mycotic Dermatitis of Cattle in British West Africa 179
COATES, HELEN, V. (See Jubb, K. V.)
COOMBS, R. R. A. (See Goodwin, R. F. W.)
COOPER, DOREEN, M., and SKULSKI, G. Effects of Feeding Furazolidone to Fowls. I. Effect on Body Weight and Sexual Development in Cockerels 299
COOPER, DOREEN, M. Effects of Feeding Furazolidone to Fowls. II. Effect on Egg Production, Fertility and Hatchability of Low Level Feeding to Pullets 312
COOPER, E. R. A., and HOWARTH, I. Some Pathological Changes in the Cat Brain 35
CUSHNIE, G. H. (See Naftalin, J. M.)

DAVIES, S. F. M. (See Joyner, L. P.)

ENTICKNAP, J. B. The Effect of Reduction of Bacterial Infiltration by Aureomycin on the Normal Alimentary Lymphoid Tissue of the Rabbit 102

FOGGIE, A. The Effect of Tick-borne Fever on the Resistance of Lambs to Staphylococci 278
FRANCIS, J. Natural and Experimental Tuberculosis in Monkeys with Observations on Immunization and Chemotherapy .. 123

GARNER, R. J. (See Blakemore, F.)
GOODWIN, R. F. W., and SAISON, RUTH. The Blood Groups of the Pig. III. A Breed Difference in Iso-Antibody Response after Vaccination with Crystal Violet Swine Fever Vaccine.. 163
GOODWIN, R. F. W., and COOMBS, R. R. A. The Blood Groups of the Pig. IV. The A Antigen-Antibody System and Haemolytic Disease in New-born Piglets 317

PAGE

HARDING, H. P., and OWEN, L. N. Eosinophilic Myositis in the Dog 109
HIRCH, ANN. Infection of Hamsters and Rabbits with *Mycobacterium johnei*. 260
HIRCH, ANN. The Aerobic Intestinal Flora in Johne's Disease .. 249
HOLMAN, H. H. (See Howell, D. G.)
HOPWOOD, JOAN B. Bacterial Flora of the Genital Tract of Ewes undergoing Caesarian Section 187
HORTON-SMITH, C., and LONG, P. L. Furazolidone in the Control of Histomoniasis (Blackhead) in Turkeys 22
HORTON-SMITH, C., and LONG, P. L. Further Observations on the Chemotherapy of Histomoniasis (Blackhead) in Turkeys 378
HOWARTH, I. (See Cooper, E. R. A.)
HOWELL, D. G., SMITH, I. M., HOLMAN, H. H., and PATTISON, I. H. Experimental Streptococcal Mastitis. XI. Immunological Studies in the Cow 49
HUDSON, J. R. (See Rowe, Barbara)

JORDAN, F. T. W. A Survey of Poultry Diseases in Mid-Wales .. 197
JOYNER, L. P., and DAVIES, S. F. M. Sulphaquinoxaline Poisoning in Chickens 39
JOYNER, L. P. (See Kendall, S. B.)
JUBB, K. V., SAUNDERS, L. Z., and COATES, HELEN, V. Thiamine Deficiency Encephalopathy in Cats 217

KENDALL, S. B., and JOYNER, L. P. The Synergism between Pyrimethamine and Sulphadimethyl-pyrimidine in the Control of *Eimeria tenella*. 145
KULASIRI, C., and SENEVIRATNE, R. D. *Gigantocotyle explanatum* Creplin (Trematoda : Paramphistomidae) Infection of the Liver of the Buffalo in Ceylon 83

LONG, P. L. (See Horton-Smith, C.)
LOVELL, R. Studies on *Corynebacterium renale*. V. Some Observations on a Chemical Extract 332

MANSI, W. Dual Vaccination of Dogs against the Canine Distemper Complex and Canine Virus Hepatitis 136
MANSI, W. (See Rowe, Barbara)
MCLEAN, SIBELY J. (See Watts, P. S.)
MICHEL, J. F. Studies on Host Resistance to Dictyocaulus Infection. II. Reinfection Experiments with *D. filaria* in Sheep 241
MICHEL, J. F. Studies on Host Resistance to Dictyocaulus Infection. III. Experiments on the Site of the Protection Mechanism 338
MOODIE, E. W. (See Stewart, J.)

NAFTALIN, J. M., and CUSHNIE, G. H. Haematology of Experimental Bracken Poisoning of Cattle. I. Changes in Blood and Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage 354

PAGE

Owen, L. N. (See Harding, H. P.)

Pattison, I. H. (See Howell, D. G.)

Reith, J. W. S. (See Stewart, J.)
Rose, J. H. The Bionomics of the Free-Living Larvae of *Dictyocaulus viviparus*. 228
Rowe, Barbara., Mansi, W., and Hudson, J. R. The Use of Fibroma Virus (Shope) for the Protection of Rabbits against Myxomatosis 290

Saison, Ruth. (See Goodwin, R. F. W.)
Saunders, L. Z. (See Jubb, K. V.)
Seneviratne, R. D. (See Kulasiri, C.)
Skulski, G. (See Cooper, Doreen M.)
Smith, H. Williams. The Chemotherapy of Experimental *Erysipelothrix rhusiopathiae* Infection in Chicks and Turkeys .. 151
Smith, I. M. (See Howell, D. G.)
Smither, A. M. (See Blaxland, J. D.)
Sojka, W. J. (See Blaxland, J. D.)
Stewart, J., and Reith, J. W. S. The Effects of Magnesian Liming on the Magnesium Content of Pasture and the Blood Level of Magnesium in Cows 1
Stewart, J., and Moodie, E. W. The Absorption of Magnesium from the Alimentary Tract of Sheep 10

Watts, P. S., and Mclean, Sibely J. Bacteroides Infection in Kangaroos 159
Wickham, Nancy. The Production and Inhibition of the Haemagglutinin of *Cl. welchii*. 62
Wickham, Nancy. The Relationship of the Haemagglutinin of *Cl. welchii* to other Soluble Antigens of this Organism .. 71

INDEX TO SUBJECTS IN VOLUME LXVI.

PAGE

ABATTOIR Material. Data on Sheep Reproduction derived from G. H. Arthur 345
Absorption of Magnesium from the Alimentary Tract of Sheep. The J. Stewart and E. W. Moodie 10
Aerobic Intestinal Flora in Johne's Disease. The Ann Hirch 249
Alimentary Lymphoid Tissue of the Rabbit. The Effect of Reduction of Bacterial Infiltration by Aureomycin on the normal J. B. Enticknap 102
Alimentary Tract of Sheep. The Absorption of Magnesium from the J. Stewart and E. W. Moodie.. 10
Antibiotics and Sulphamezathine on the Causal Agent of Virus Pneumonia of Pigs. The Action of A. O. Betts and R. C. Campbell.. 89
Antibodies in the Bovine. The Maternal Transference of F. Blakemore and R. J. Garner 287
Antigen-Antibody System and Haemolytic Disease in New-born Piglets. The Blood Groups of the Pig. IV. The A R. F. W. Goodwin and R. R. A. Coombs 317
Aureomycin on the Normal Alimentary Lymphoid Tissue of the Rabbit. The Effect of Reduction of Bacterial Infiltration by J. B. Enticknap 102

BACTERIAL Flora of the Genital Tract of Ewes undergoing Caesarian Section. Joan B. Hopwood 187
Bacterial Infiltration by Aureomycin on the Normal Alimentary Lymphoid Tissue of the Rabbit. The Effect of Reduction of J. B. Enticknap 102
Bacteroides Infection in Kangaroos. P. S. Watts and Sibely J. McLean 159
Bionomics of the Free-Living Larvae of *Dictyocaulus viviparus*. The J. H. Rose 228
(Blackhead) in Turkeys. Furazolidone in the Control of Histomoniasis C. Horton-Smith and P. L. Long 22
(Blackhead) in Turkeys. Further observations on the Chemotherapy of Histomoniasis C. Horton-Smith and P. L. Long 378
Blood and Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage. Haematology of Experimental Bracken Poisoning of Cattle. I. Changes in J. M. Naftalin and G. H. Cushnie 354
Blood Groups of the Pig. III. A Breed Difference in Iso-Antibody Response after Vaccination with Crystal Violet Swine Fever Vaccine. The R. F. W. Goodwin and Ruth Saison .. 163
Blood Groups of the Pig. IV. The A Antigen-Antibody System and Haemolytic Disease in New-born Piglets. The R. F. W. Goodwin and R. R. A. Coombs 317
Blood Level of Magnesium in Cows. The Effects of Magnesian Liming on the Magnesium Content of Pasture and the J. Stewart and J. W. S. Reith 1

PAGE

Body-Weight and Sexual Development in Cockerels. Effects of Feeding Furazolidone to Fowls. I. Effect on Doreen M. Cooper and G. Skulski 299

Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage. Haematology of Experimental Bracken Poisoning of Cattle. I. Changes in Blood and J. M. Naftalin and G. H. Cushnie 354

Bracken Poisoning of Cattle. I. Changes in Blood and Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage. Haematology of Experimental J. M. Naftalin and G. H. Cushnie 354

Brain. Some Pathological Changes in the Cat E. R. A. Cooper and I. Howarth.. 35

Breed Difference in Iso-Antibody Response after Vaccination with Crystal Violet Swine Fever Vaccine. The Blood Groups of the Pig. III. A R. F. W. Goodwin and Ruth Saison 163

Buffalo in Ceylon. *Gigantocotyle explanatum* Creplin (Trematoda : Paramphistomidae) Infection of the Liver of the C. Kulasiri and R. D. Seneviratne 83

CAESARIAN Section. Bacterial Flora of the Genital Tract of Ewes undergoing Joan B. Hopwood 187

Canine Distemper Complex and Canine Virus Hepatitis. Dual Vaccination of Dogs against the W. Mansi 136

Canine Virus Hepatitis. Dual Vaccination of Dogs against the Canine Distemper Complex and W. Mansi 136

Cat Brain. Some Pathological Changes in the E. R. A. Cooper and I. Howarth.. 35

Cats. Thiamine Deficiency Encephalopathy in K. V. Jubb, L. Z. Saunders and Helen V. Coates 217

Cattle in British West Africa. Mycotic Dermatitis of K. S. Chodnik 179

Cattle. I. Changes in Blood and Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage. Haematology of Experimental Bracken Poisoning of J. M. Naftalin and G. H. Cushnie 354

Cell Inclusions in Swine Pox. Morphology of the Elementary Bodies and F. Blakemore and M. Abdussalam 373

Ceylon. *Gigantocotyle explanatum* Creplin (Trematoda : Paramphistomidae) Infection of the Liver of the Buffalo in C. Kulasiri and R. D. Seneviratne 83

Chemical Extract. Studies on *Corynebacterium renale*. V. Some Observations on a R. Lovell 332

Chemotherapy. Natural and Experimental Tuberculosis in Monkeys with Observations on Immunization and J. Francis 123

Chemotherapy of Experimental *Erysipelothrix rhusiopathiae* Infection in Chicks and Turkeys. The H. Williams Smith .. 151

Chemotherapy of Histomoniasis (Blackhead) in Turkeys. Further Observations on the C. Horton-Smith and P. L. Long .. 378

Chickens. Sulphaquinoxaline Poisoning in L. P. Joyner and S. F. M. Davies 39

PAGE

Chicks and Turkeys. The Chemotherapy of Experimental *Erysipelothrix rhusiopathiae* Infection in H. Williams Smith 151
Cl. welchii. The Production and Inhibition of the Haemagglutinin of Nancy Wickham 62
Cl. welchii to other Soluble Antigens of this Organism. The Relationship of the Haemagglutinin of Nancy Wickham 71
Cockerels. Effects of Feeding Furazolidone to Fowls. I. Effect on Body-Weight and Sexual Development in Doreen M. Cooper and G. Skulski 299
Corynebacterium renale. V. Some Observations on a Chemical Extract. Studies on R. Lovell 332
Cows. The Effects of Magnesian Liming on the Magnesium Content of Pasture and the Blood Level of Magnesium in J. Stewart and J. W. S. Reith 1

DEFICIENCY Encephalopathy in Cats. Thiamine K. V. Jubb, L. Z. Saunders and Helen V. Coates 217
Dermatitis of Cattle in British West Africa. Mycotic K. S. Chodnik 179
Dictyocaulus Infection. II. Reinfection Experiments with *D. filaria* in Sheep. Studies on Host Resistance to J. F. Michel 241
Dictyocaulus Infection. III. Experiments on the Site of the Protective Mechanism. Studies on Host Resistance to J. F. Michel.. 338
Dictyocaulus viviparus. The Bionomics of the Free-Living Larvae of J. H. Rose 228
Distemper Complex and Canine Virus Hepatitis. Dual Vaccination of Dogs against the Canine W. Mansi 136
Dog. Eosinophilic Myositis in the H. P. Harding and L. N. Owen 109
Dogs against the Canine Distemper Complex and Canine Virus Hepatitis. Dual Vaccination of. W. Mansi 136
Dual Vaccination of Dogs against the Canine Distemper Complex and Canine Virus Hepatitis. W. Mansi 136

EGG Production, Fertility and Hatchability of Low Level Feeding to Pullets. Effects of Feeding Furazolidone to Fowls. II. Effect on Doreen M. Cooper 312
Eimeria tenella. The Synergism between Pyrimethamine and Sulphadimethyl-pyrimidine in the Control of S. B. Kendall and L. P. Joyner 145
Elementary Bodies and Cell Inclusions in Swine Pox. Morphology of the F. Blakemore and M. Abdussalam 373
Encephalopathy in Cats. Thiamine Deficiency K. V. Jubb, L. Z. Saunders and Helen V. Coates 217
Eosinophilic Myositis in the Dog. H. P. Harding and L. N. Owen 109

Erysipelothrix rhusiopathiae Infection in Chicks and Turkeys. The Chemotherapy of Experimental H. Williams Smith .. 151
Ewes undergoing Caesarian Section. Bacterial Flora of the Genital Tract of Joan B. Hopwood 187

PAGE

FERTILITY and Hatchability of Low Level Feeding to Pullets. Effects of Feeding Furazolidone to Fowls. II. Effect on Egg Production Doreen M. Cooper 312
Fibroma Virus (Shope) for the Protection of Rabbits against Myxomatosis. The Use of Barbara Rowe, W. Mansi and J. R. Hudson 290
Field Outbreaks of Disease. A Study of *Salm. pullorum* and *Salm. gallinarum* Strains isolated from J. D. Blaxland, W. J. Sojka and A. M. Smither 270
Fowls. I. Effect on Body-Weight and Sexual Development in Cockerels. Effects of Feeding Furazolidone to Doreen M. Cooper and G. Skulski 299
Fowls. II. Effect on Egg Production, Fertility and Hatchability of Low Level Feeding to Pullets. Effects of Feeding Furazolidone to Doreen M. Cooper 312
Free-Living Larvae of *Dictyocaulus viviparus*. The Bionomics of the J. H. Rose 228
Furazolidone in the Control of Histomoniasis (Blackhead) in Turkeys. C. Horton-Smith and P. L. Long 22
Furazolidone to Fowls. I. Effect on Body-Weight and Sexual Development in Cockerels. Effects of Feeding Doreen M. Cooper and G. Skulski 299
Furazolidone to Fowls. II. Effect on Egg Production, Fertility and Hatchability of Low Level Feeding to Pullets. Effects of Feeding Doreen M. Cooper 312

GENITAL tract of Ewes undergoing Caesarian Section. Bacterial Flora of the Joan B. Hopwood 187
Gigantocotyle explanatum Creplin (Trematoda : Paramphistomidae) Infection of the Liver of the Buffalo in Ceylon. C. Kulasiri and R. D. Seneviratne 83

HAEMAGGLUTININ of *Cl. welchii*. The Production and Inhibition of the Nancy Wickham 62
Haemagglutinin of *Cl. welchii* to other Soluble Antigens of this Organism. The Relationship of the Nancy Wickham .. 71
Haematology of Experimental Bracken Poisoning of Cattle. I. Changes in Blood and Bone Marrow. II. Attempts to Modify the Course of the Bone Marrow Damage. J. M. Naftalin and G. H. Cushnie 354
Haemolytic Disease in New-Born Piglets. The Blood Groups of the Pig. IV. The A Antigen-Antibody System and R. F. W. Goodwin and R. R. A. Coombs. 317
Hamsters and Rabbits with *Mycobacterium johnei*. Infection of Ann Hirch 260
Hatchability of Low Level Feeding to Pullets. Effects of Feeding Furazolidone to Fowls. II. Effect on Egg Production, Fertility and Doreen M. Cooper 312
Hepatitis. Dual Vaccination of Dogs against the Canine Distemper Complex and Canine Virus W. Mansi 136

PAGE

Histomoniasis (Blackhead) in Turkeys. Furazolidone in the Control of C. Horton-Smith and P. L. Long 22
Histomoniasis (Blackhead) in Turkeys. Further Observations on the Chemotherapy of C. Horton-Smith and P. L. Long 378
Host Resistance to Dictyocaulus Infection. II. Reinfection Experiments with *D. filaria* in Sheep. Studies on J. F. Michel 241
Host Resistance to Dictyocaulus Infection. III. Experiments on the Site of the Protection Mechanism. Studies on J. F. Michel 338

IMMUNIZATION and Chemotherapy. Natural and Experimental Tuberculosis in Monkeys with Observations on J. Francis 123
Immunological Studies in the Cow. Experimental Streptococcal Mastitis. XI. D. G. Howell, I. M. Smith, H. H. Holman and I. H. Pattison 49
Inclusions in Swine Pox. Morphology of the Elementary Bodies and Cell F. Blakemore and M. Abdussalam 373
Inhibition of the Haemagglutinin of *Cl. welchii*. The Production and Nancy Wickham 62
Intestinal Flora in Johne's Disease. The Aerobic Ann Hirch 249
Iso-Antibody Response after Vaccination with Crystal Violet Swine Fever Vaccine. The Blood Groups of the Pig. III. A Breed Difference in R. F. W. Goodwin and Ruth Saison 163
JOHNE'S Disease. The Aerobic Intestinal Flora in. Ann Hirch 249

KANGAROOS. Bacteroides Infection in P. S. Watts and Sibely J. McLean 159

LAMBS to Staphylococci. The Effect of Tick-borne Fever on the Resistance of A. Foggie 278
Larvae of *Dictyocaulus viviparus*. The Bionomics of the Free-Living J. H. Rose 228
Liver of the Buffalo in Ceylon. *Gigantocotyle explanatum* Creplin (Trematoda : Paramphistomidae) Infection of the C. Kulasiri and R. D. Seneviratne 83
Lymphoid Tissue of the Rabbit. The Effect of Reduction of Bacterial Infiltration by Aureomycin on the Normal Alimentary J. B. Enticknap 102

MAGNESIAN Liming on the Magnesium Content of Pasture and the Blood Level of Magnesium in Cows. The Effects of J. Stewart and J. W. S. Reith 1
Magnesium Content of Pasture and the Blood Level of Magnesium in Cows. The Effects of Magnesian Liming on the J. Stewart and J. W. S. Reith 1
Magnesium from the Alimentary Tract of Sheep. The Absorption of J. Stewart and E. W. Moodie 10
Mastitis. XI. Immunological Studies in the Cow. Experimental Streptococcal D. G. Howell, I. M. Smith, H. H. Holman and I. H. Pattison 49

PAGE

Maternal Transference of Antibodies in the Bovine. The F. Blakemore and R. J. Garner 287
Monkeys, with Observations on Immunization and Chemotherapy. Natural and Experimental Tuberculosis in J. Francis .. 123
Morphology of the Elementary Bodies and Cell Inclusions in Swine Pox. F. Blakemore and M. Abdussalam 373
Mycobacterium johnei. Infection of Hamsters and Rabbits with Ann Hirch 260
Mycotic Dermatitis of Cattle in British West Africa. K. S. Chodnik 179
Myositis in the Dog. Eosinophilic H. P. Harding and L. N. Owen 109
Myxomatosis. The Use of Fibroma Virus (Shope) for the Protection of Rabbits against Barbara Rowe, W. Mansi and J. R. Hudson 290

NATURAL and Experimental Tuberculosis in Monkeys with Observations on Immunization and Chemotherapy. J. Francis 123
New-born Piglets. The Blood Groups of the Pig. IV. The A Antigen-Antibody System and Haemolytic Disease in R. F. W. Goodwin and R. R. A. Coombs 317

PASTURE and the Blood Level of Magnesium in Cows. The Effects of Magnesian Liming on the Magnesium Content of J. Stewart and J. W. S. Reith 1
Pathological Changes in the Cat Brain. Some E. R. A. Cooper and I. Howarth 35
Pig. III. A Breed Difference in Iso-Antibody Response after Vaccination with Crystal Violet Swine Fever Vaccine. The Blood Groups of the R. F. W. Goodwin and Ruth Saison 163
Piglets. The Blood Groups of the Pig. IV. The A Antigen-Antibody System and Haemolytic Disease in New-born R. F. W. Goodwin and R. R. A. Coombs 317
Pigs. The Action of Antibiotics and Sulphamezathine on the Causal Agent of Virus Pneumonia of A. O. Betts and R. C. Campbell 89
Pneumonia of Pigs. The Action of Antibiotics and Sulphamezathine on the Causal Agent of Virus A. O. Betts and R. C. Campbell 89
Poisoning in Chickens. Sulphaquinoxaline L. P. Joyner and S. F. M. Davies 39
Poisoning of Cattle. I. Changes in the Blood and Bone Marrow. II. Attempts to modify the Course of the Bone Marrow Damage. Haematology of Experimental Bracken J. M. Naftalin and G. H. Cushnie 354
Poultry Diseases in Mid-Wales. A Survey of F. T. W. Jordan 197
Production and Inhibition of the Haemagglutinin of *Cl. welchii*. The Nancy Wickham 62
Protection of Rabbits against Myxomatosis. The Use of Fibroma Virus (Shope) for the Barbara Rowe, W. Mansi and J. R. Hudson 290

PAGE

Pullets. Effects of Feeding Furazolidone to Fowls. II. Effect on Egg Production, Fertility and Hatchability of Low Level Feeding to Doreen M. Cooper 312
Pyrimethamine and Sulphadimethyl-pyrimidine in the Control of *Eimeria tenella*. The Synergism between S. B. Kendall and L. P. Joyner 145

RABBIT. The Effect of Reduction of Bacterial Infiltration by Aureomycin on the Normal Alimentary Lymphoid Tissue of the J. B. Enticknap 102
Rabbits with *Mycobacterium johnei*. Infection of Hamsters and Ann Hirch 260
Rabbits against Myxomatosis. The Use of Fibroma Virus (Shope) for the Protection of Barbara Rowe, W. Mansi and J. R. Hudson 290
Reproduction derived from Abattoir Material. Data on Sheep G. H. Arthur 345
Resistance to Dictyocaulus Infection. II. Reinfection Experiments with *D. filaria* in Sheep. Studies on Host J. F. Michel.. 241
Resistance to Dictyocaulus Infection. III. Experiments on the Site of the Protective Mechanism. Studies on Host J. F. Michel 338
Resistance of Lambs to Staphylococci. The Effect of Tick-borne Fever on the A. Foggie 278

Salm. gallinarum Strains Isolated from Field Outbreaks of Disease. A Study of *Salm. pullorum* and J. D. Blaxland, W. J. Sojka and A. M. Smither 270
Salm. pullorum and *Salm. gallinarum* Strains Isolated from Field Outbreaks of Disease. A Study of J. D. Blaxland, W. J. Sojka and A. M. Smither 270
Sexual Development in Cockerels. Effects of Feeding Furazolidone to Fowls. I. Effect on Body Weight and Doreen M. Cooper and G. Skulski 299
Sheep. The Absorption of Magnesium from the Alimentary Tract of J. Stewart and E. W. Moodie.. 10
Sheep Reproduction derived from Abattoir Material. Data on G. H. Arthur 345
Sheep. Studies on Host Resistance to Dictyocaulus Infection. II. Reinfection Experiments with *D. filaria* in J. F. Michel 241
(Shope) for the Protection of Rabbits against Myxomatosis. The Use of Fibroma Virus Barbara Rowe, W. Mansi and J. R. Hudson 290
Staphylococci. The Effect of Tick-borne Fever on the Resistance of Lambs to A. Foggie 278
Streptococcal Mastitis. XI. Immunological Studies in the Cow. Experimental D. G. Howell, I. M. Smith, H. H. Holman and I. H. Pattison.. 49
Sulphadimethyl-pyrimidine in the Control of *Eimeria tenella*. The Synergism between Pyrimethamine and S. B. Kendall and L. P. Joyner 145

PAGE

Sulphamezathine on the Causal Agent of Virus Pneumonia of Pigs. The Action of Antibiotics and A. O. Betts and R. C. Campbell 89
Sulphaquinoxaline Poisoning in Chickens. L. P. Joyner and S. F. M. Davies 39
Survey of Poultry Diseases in Mid-Wales. A F. T. W. Jordan 197
Swine Fever Vaccine. The Blood Groups of the Pig. III. A Breed Difference in Iso-Antibody Response after Vaccination with Crystal Violet R. F. W. Goodwin and Ruth Saison .. 163
Swine Pox. Morphology of the Elementary Bodies and Cell Inclusions in F. Blakemore and M. Abdussalam.. .. 373
Synergism between Pyrimethamine and Sulphadimethyl-pyrimidine in the Control of *Eimeria tenella*. The S. B. Kendall and L. P. Joyner.. 145

THIAMINE Deficiency Encephalopathy in Cats. K. V. Jubb, L. Z. Saunders and Helen V. Coates 217
Tick-borne Fever on the Resistance of Lambs to Staphylococci. The Effect of A. Foggie 278
(Trematoda : Paramphistomidae). Infection of the Liver of the Buffalo in Ceylon. *Gigantocotyle explanatum* Creplin C. Kulasiri and R. D. Seneviratne 83
Tuberculosis in Monkeys with Observations on Immunization and Chemotherapy. Natural and Experimental J. Francis.. 123
Turkeys. The Chemotherapy of Experimental *Erysipelothrix rhusiopathiae* Infection in Chicks and H. Williams Smith 151
Turkeys. Furazolidone in the Control of Histomoniasis (Blackhead) in C. Horton-Smith and P. L. Long 22
Turkeys. Further Observations on the Chemotherapy of Histomoniasis (Blackhead) in. C. Horton-Smith and P. L. Long 378

VACCINATION with Crystal Violet Swine Fever Vaccine. The Blood Groups of the Pig. III. A Breed Difference in Iso-Antibody Response after R. F. W. Goodwin and Ruth Saison 163
Vaccination of Dogs against the Canine Distemper Complex and Canine Virus Hepatitis. Dual W. Mansi 136
Virus Hepatitis. Dual Vaccination of Dogs against the Canine Distemper Complex and Canine W. Mansi 136
Virus Pneumonia of Pigs. The Action of Antibiotics and Sulphamezathine on the Causal Agent of A. O. Betts and R. C. Campbell 89
Virus (Shope) for the Protection of Rabbits against Myxomatosis. The Use of Fibroma Barbara Rowe, W. Mansi and J. R. Hudson 290

WALES. A Survey of Poultry Diseases in Mid- F. T. W. Jordan 197

West Africa. Mycotic Dermatitis of Cattle in British K. S. Chodnik 179

THE EFFECTS OF MAGNESIAN LIMING ON THE MAGNESIUM CONTENT OF PASTURE AND THE BLOOD LEVEL OF MAGNESIUM IN COWS

By

J. Stewart

Moredun Institute, Gilmerton, Edinburgh

and

J. W. S. Reith

Macaulay Institute for Soil Research, Aberdeen

INTRODUCTION

Hypomagnesaemic tetany or "lactation tetany" of cattle has never been classified as a true deficiency disease caused by a deficiency of magnesium in the pasture or diet by any of the workers in this field of research. Blakemore and Stewart (1934-35) found that the magnesium content of various pastures from farms where the disease had been prevalent was no less, and in some instances was even higher, than on neighbouring farms on which it had never been observed. They concluded, "Its (lactation tetany) occurrence cannot be explained on the ground of variation in the magnesium content of the herbage." No other workers have yet published data which contradict this statement, but the magnesium content of crops and herbage has not received much attention from soil or plant chemists in Britain until recently when Reith (1955) showed that the magnesium content of mixed herbage increased by 60 per cent to 100 per cent from April to August; the values being relatively low during April, May and early June, and gradually increasing until August after which they remained reasonably constant. Thus, despite little difference between the magnesium content of pasture on which hypomagnesaemia occurs and that on which it has never been observed, the "Spring type of occurrence" of hypomagnesaemic tetany (Stewart, 1954) which is the most common type, coincides with the time of year when the concentration of magnesium in pasture is at its lowest. Reith (1955) also showed, in experiments carried out over a three year period, that magnesian limestone dressings increased the magnesium content of mixed herbage throughout the growing season by at least 50 per cent above that of similar herbage receiving no lime dressings or ordinary limestone dressings. Such an increase in the magnesium content of pasture in April and May will increase the magnesium intake of the animals grazing it by about 20 g. MgO per day, assuming that a dairy cow will eat 30 lb. of dry matter per day and that the pasture content of MgO has increased from 0.30 per cent to 0.45 per cent. This is comparable to the amount of magnesium each cow obtains when fed 5 oz. per day of a high magnesium mineral mixture containing 16 per cent MgO as suggested by Blakemore and Stewart (1934), and shown by them to

reduce the degree of hypomagnesaemia and the risk of tetany in herds put to pasture in April and May.

PRESENT INVESTIGATION

In view of the above findings it was considered desirable to ascertain if the increased content of magnesium of pasture dressed with magnesian limestone would prevent the seasonal hypomagnesaemia on farms where tetany was prevalent.

Farm A was selected since many cases of tetany had occurred in April and May, 1948. Blood samples had been taken in April, June and July, 1948, the magnesium contents of these samples being shown in Table 1. The normal level for the magnesium content of the blood of cows is 2.5 to 3.0 mg. Mg per 100 ml. serum with 2.0 mg. Mg as the lower limit of the normal range. From Table 1 it will be seen that in April, when cases of tetany were occurring,

TABLE 1

MAGNESIUM CONTENT OF BLOOD OF COWS ON FARM A IN 1948

mg. Mg. per 100 ml. serum

No.	*April 1948*	*June 1948*	*July 1948*
1	1·82	2·19	2·47
2	1·34	2·35	2·28
3	1·45	2·46	2·61
4	1·79	2·40	2·61
5	2·12	2·33	2·71
6	1·96	2·30	2·63
7	1·35	2·53	2·78
8	2·01	2·38	2·60
9	1·72	2·25	2·37
10	1·53	2·62	2·53
Mean	1·71	2·38	2·56

a general hypomagnesaemia was present throughout the herd and there was a marked increase in the blood magnesium level by June but no real difference between June and July values. This is comparable to what is usually found in herds in hypomagnesaemic tetany districts.

In April, 1949 a dressing of 40 cwt. per acre magnesian limestone containing 20 per cent MgO and 31 per cent CaO was applied to a ploughed field on Farm A, with the exception of one part of the field which was reserved for an experiment to provide pasture samples for analyses in subsequent years. This experimental area was divided into a number of plots and the three main treatments, applied in April, 1949, were:

No lime

40 cwt. per acre ground limestone (48 per cent CaO + 2 per cent MgO)

40 cwt. per acre ground magnesian limestone (20 per cent MgO + 31 per cent CaO)

TABLE 2

EFFECT OF LIME TREATMENT ON THE MAGNESIUM CONTENT OF PASTURE AT FARM A

%Mg. in oven-dry herbage

Date of sampling	No lime				40 cwt. ground limestone per acre				40 cwt. ground magnesian limestone per acre			
	1950	1951	1952	Mean	1950	1951	1952	Mean	1950	1951	1952	Mean
25th to 30th April ..	0·17	0·18	0·17	0·17	0·17	0·21	0·18	0·19	0·29	0·31	0·30	0·30
15th to 21st May ..	0·18	0·14	0·17	0·16	0·19	0·16	0·17	0·17	0·30	0·23	0·26	0·26
31st May to 5th June ..	0·20	0·16	0·18	0·18	0·20	0·20	0·19	0·20	0·32	0·25	0·28	0·28
15th to 20th June ..	0·23	0·16	0·22	0·20	0·20	0·17	0·24	0·20	0·33	0·29	0·26	0·29
3rd to 10th July ..	0·25	0·23	0·22	0·23	0·28	0·23	0·28	0·26	0·42	0·38	0·35	0·38
19th to 24th July ..	0·31	0·22	0·20	0·24	0·32	0·24	0·19	0·25	0·48	0·34	0·30	0·37
10th to 16th August ..	0·26	0·22	0·24	0·24	0·29	0·26	0·25	0·27	0·47	0·39	0·34	0·40
6th to 8th September ..	0·28	0·24	0·25	0·26	0·30	0·25	0·26	0·27	0·42	0·34	0·35	0·37
10th to 27th October ..	0·29	0·26	0·23	0·26	0·31	0·29	0·23	0·28	0·42	0·43	0·34	0·40
23rd November ..	—	0·23	—	—	—	0·25	—	—	—	0·36	—	—
Mean	0·24	0·20	0·21	0·22	0·25	0·22	0·22	0·23	0·38	0·33	0·31	0·34

The whole field including the experimental plots was planted with oats undersown with grass and clover seeds, a normal dressing of fertilizer being applied broadcast. In the spring of 1950 the experimental plots were fenced off and throughout the growing seasons of 1950, 1951 and 1952, the herbage was sampled regularly at intervals of two to four weeks depending on the amount of growth. The experimental plots received dressings at the rate of $1\frac{1}{2}$ cwt. nitro chalk and 4 cwt. potassic superphosphate (15 per cent P_2O_5, 10 per cent K_2O) per acre in April each year, and the rest of the field was similarly treated. After each sampling the grass was cut and removed in order to obtain herbage similar to that which cows would be grazing in fields. The magnesium contents of the samples are given in Table 2.

These results show the seasonal variation in the magnesium content of mixed herbage and the marked increase in it produced by applying magnesian limestone, referred to by Reith (1955). The magnesian limestone dressing increased the percentage of magnesium in the herbage by over 50 per cent. The herbage from the magnesian limestone treated plots may be regarded as being quite typical of the produce in the treated field. In order to rest this treated field at intervals, in February 1950 another grass field was treated with 30 cwt. per acre ground magnesian limestone (15 per cent MgO and 43 per cent CaO). These two fields provided all the grazing for the experimental cows during the 1950 to 1952 period.

As the investigation was being carried out with a commercial dairy herd it was difficult to achieve an ideally designed and perfectly controlled experiment, since several fields of grass had to be reserved at the critical weeks in April and May for the making of grass silage, and this reduced the number of fields available for the apportioning of the herd between treated and untreated fields during the summer months. It was a common practice on this farm to utilise the pastures of two other neighbouring farms, B and C, owned by the farmer, for members of the herd, and we had to use as our control cows in 1949, 1950 and 1951 those pastured on these farms but still members of the same herd, of the same breeding and of the same management during the winter months. The control cows grazed on farm B in 1949 and 1950 and on farm C in 1951. Hypomagnesaemic tetany was also very prevalent on these farms. In 1952 we were forced to use as controls the cows on a farm, D, which had a similar history over 1949 to 1952 of hypomagnesaemic tetany, and for which in 1952 no type of therapy was being used.

Samples of herbage were taken from the fields grazed by the control cows on farms B, C and D during 1949, 1951 and 1952 but not from the field of farm B in 1950 since it was presumed that the magnesium content of the herbage would be very similar to that of 1949. The magnesium contents of these samples (Table 3) were similar to those obtained from the no-lime- and ground-limestone-

treatment samples in the fenced experimental plot on farm A as shown in Table 2. It can be assumed, therefore, that the control cows throughout all the years of experiment were grazing herbage of a magnesium content similar to what they would have obtained

TABLE 3

MAGNESIUM CONTENT OF PASTURE ON FARMS B, C, AND D

	Date of sampling	*% Mg. oven dried material*
Farm B	29.4.49	0·17
	18.5.49	0·24
	1.6.49	0·23
	13.6.49	0·23
	14.7.49	0·38
	15.8.49	0·30
Farm C	27.4.51	0·19
	18.5.51	0·14
	5.6.51	0·13
	20.6.51	0·24
	6.7.51	0·22
	24.7.51	0·23
	16.8.51	0·24
	7.9.51	0·22
	11.10.51	0·34
Farm D	14.5.52	0·16
	4.6.52	0·12
	20.6.52	0·22
	8.7.52	0·23
	24.7.52	0·22
	12.8.52	0·22
	8.9.52	0·27
	15.10.52	0·26

on the field of farm A if no magnesian limestone dressings had been spread.

In 1949 twelve cows were selected and bled in February whilst still housed and being fed winter rations. They were again bled in April after having been at pasture for over two weeks, and again in May and June. A similar procedure was carried out in 1950, 1951 and 1952—the twelve cows being bled in either February or March whilst still housed, and then in April, after a fortnight on the magnesian-limestone-dressed pasture. Further bleedings were taken in May and again in June or July while the animals were still confined to the magnesian-limestone-dressed pasture. Care was taken to ensure that on return to the pasture after each milking the cows were placed in the correct fields. The wear and tear of a commercial dairy herd only allowed of six of the original twelve animals remaining in the experiment throughout the period 1949 to 1952 and these are numbered 1 to 6 in Table 4. The other six animals bled at each sampling are labelled A to F, but are not necessarily the same cows in different years, although for any one

TABLE 4

MAGNESIUM CONTENT OF BLOOD OF COWS OF FARM A

mg. Mg. per 100 ml. serum

No.	*1949*				*1950*				*1951*				*1952*			
	Feb.	*April*	*May*	*June*	*Feb.*	*April*	*May*	*June*	*March*	*April*	*May*	*July*	*March*	*April*	*May*	*July*
1	2·65	1·53	2·63	2·59	2·54	2·01	2·61	3·08	2·98	2·21	2·42	2·34	2·78	2·21	2·56	2·66
2	2·42	2·72	2·52	3·17	2·01	2·34	2·59	3·05	2·32	2·41	2·56	2·44	2·83	2·32	2·42	2·78
3	2·81	2·10	2·54	2·69	2·42	2·19	2·52	3·17	2·82	1·99	2·17	2·96	2·68	2·08	2·29	2·51
4	2·60	1·58	3·07	2·52	2·26	1·96	2·48	2·61	2·51	2·08	2·30	2·38	2·56	2·00	2·08	2·23
5	3·17	1·64	2·67	2·64	2·30	2·18	2·64	2·60	2·72	2·23	2·48	2·36	2·92	2·24	2·26	2·41
6	2·31	1·72	2·75	2·67	2·75	1·86	3·00	2·45	2·43	2·18	2·09	2·17	3·01	2·37	2·34	2·65
Mean (1–6) ..	2·66	1·88	2·70	2·71	2·38	2·09	2·64	2·83	2·63	2·18	2·34	2·44	2·80	2·20	2·32	2·54
A	2·74	1·48	2·61	2·45	2·98	1·92	2·69	3·17	2·69	1·76	2·47	2·46	2·56	2·46	2·62	2·70
B	2·67	2·35	2·38	2·58	2·56	2·38	2·59	1·96	2·55	1·91	2·38	2·68	2·72	2·32	2·56	2·43
C	2·85	1·83	2·71	2·79	2·52	2·41	2·34	2·59	2·72	2·42	2·19	2·12	2·31	1·98	2·08	2·28
D	2·67	1·19	2·52	2·49	2·27	2·32	2·38	2·24	3·07	2·76	2·78	2·74	2·72	1·86	2·32	2·46
E	2·87	1·92	2·41	2·58	2·71	1·75	2·29	2·38	2·75	2·48	3·02	2·96	2·52	2·41	2·19	2·39
F	2·46	1·36	2·36	2·62	2·54	1·82	2·19	2·41	2·67	1·99	2·53	2·72	2·43	1·86	2·48	2·62
Mean (A–F) ..	2·71	1·69	2·49	2·59	2·59	2·10	2·41	2·46	2·74	2·22	2·56	2·61	2·54	2·15	2·37	2·48

		Feb./March	*April*	*May*	*June/July*
Mean of means	Cows 1–6	2·62	2·09	2·50	2·63
	Cows A–F	2·65	2·04	2·46	2·53
	Cows 1–6 & A–F ..	2·63	2·06	2·48	2·58

Standard Error of Means

Cows 1–6 ± 0·087
Cows A–F ± 0·050
Cows 1–6 & A–F ± 0·034

year the same animals remained on experiment throughout and so help to reflect the status of the magnesium content of the blood of the herd for that year. The magnesium values of the blood samples are shown in Table 4.

In an analysis of variance the residual variance of Cows 1 to 6 was 0.0458 with 44 degrees of freedom and for Cows A to F 0.0599 with 64 degrees of freedom—thus the two variances were homogeneous. Since for the two groups there is no significant difference between variances or between the monthly means this indicates that both groups can be regarded as belonging to the same population and equally representative of the herd under investigation.

The mean values for the months of sampling show a marked fall in April compared with the other months. The degree of hypomagnesaemia in 1949 was comparable to that of 1948 (Table 1) and was significantly greater ($P<0.01$) than in 1950, 1951 and 1952. This would suggest that the magnesian limestone dressing which had significantly increased the magnesium content of the herbage (Table 2) had also been instrumental in reducing the degree of hypomagnesaemia in the herd grazing the treated herbage. It is of interest to record that no cases of tetany occurred on farm A in 1951 and 1952 when the majority of the herd were on magnesium-dressed pasture.

The magnesium values for the May samples show that recovery had not been completed by the date of sampling as the mean value for that month was significantly less than for February/March and June/July, though much higher than for April. Thus whilst the magnesium content of the herbage was still low there was some degree of recovery to a normal level of blood magnesium. This is further proof that it is not primarily the magnesium content of the diet which causes the hypomagnesaemia or the upset of magnesium metabolism. Experiments described by Bartlett, Brown, Foot, Rowland, Allcroft and Parr (1954) in which milking cows were turned out to small plots under different manuring treatments, one of which was 25 cwt. magnesite + 6 cwt. sulphate of ammonia per acre, revealed that such a dressing also prevented severe hypomagnesaemia. These authors considered, however, that it was more likely to have been the clover content of the sward than its magnesium content which prevented the hypomagnesaemia as the hypomagnesaemia was almost entirely confined to conditions where little clover was present.

Blood samples were taken from six control cows (see above) each year on the same day in the month of April as the samples from the experimental cows on farm A. The magnesium contents of these samples are shown in Table 5.

Statistical analysis showed that the variances within each set of six samples were homogeneous and that there were no significant differences between the mean values for the four sets.

Comparing the April values of Table 4 it can be seen that the

April 1949 mean does not differ from the overall mean of Table 5, but that the means for April of 1950, 1951 and 1952 are significantly greater than it, P being less than 0.01 in each case. If therefore it can be assumed that the four farms and the different sets of cows are comparable, and we believe they are, this confirms the suggestion

TABLE 5

MAGNESIUM CONTENT OF BLOOD OF CONTROL COWS ON FARMS B, C, AND D

(mg. Mg. per 100 ml. of serum)

No.	*Farm B*		*Farm C*	*Farm D*
	April 1949	*April 1950*	*April 1951*	*April 1952*
1	1·47	2·31	1·32	1·96
2	1·86	1·76	1·58	1·32
3	2·03	1·64	2·41	1·78
4	2·10	1·20	1·98	1·52
5	1·42	1·79	1·62	2·22
6	1·54	2·06	2·17	1·78
Mean	1·74	1·79	1·85	1·76

Overall mean value = 1·785 ± 0·0722

Standard Error of mean ± 0·144

that the magnesian limestone dressing had increased the blood magnesium on farm A in 1950, 1951 and 1952.

The results obtained in this investigation were similar to those of Blakemore and Stewart (1934-1935) in their experiments on the effects of feeding high magnesium mineral mixtures to cattle. The lessening of the degree of hypomagnesaemia in April seemed sufficient to sustain the blood magnesium above the level of potential cases of tetany.

Although from the results of this investigation it cannot be definitely asserted that dressings of magnesian limestone will prevent hypomagnesaemic tetany, yet in the light of our present knowledge of the aetiology of this condition any farm procedure which will increase the magnesium content of the diet of cattle in April and so sustain the magnesium content of the blood at the highest possible level is to be recommended.

CONCLUSIONS

Recent work has shown that there is a large seasonal variation in the magnesium content of herbage, the lowest content being in April and May when magnesium tetany is most prevalent. It has also been shown that dressings of magnesian limestone spread on the herbage can increase the magnesium content by over 75 per cent. Experiments described in this paper suggest that the higher magnesium content of the herbage in April and May produced by

magnesian limestone dressings considerably reduced the degree of hypomagnesaemia in a herd of cows on a farm where magnesium tetany was prevalent.

ACKNOWLEDGMENT

The authors wish to thank Mr. R. H. E. Inkson, Aberdeen University, for help in the statistical examination of the data.

REFERENCES

Bartlett, S., Brown, B. B., Foot, A. S., Rowland, S. J., Allcroft, R., and Parr, W. H. (1954). *Brit. vet. J.*, **110**, 3.
Blakemore, F., and Stewart, J. (1934-35). *Rep. Inst. anim. Path. Camb.*, **4**, 103.
Reith, J. W. S. (1955). *Emp. J. exp. Agric.* (in the press).
Stewart, J. (1954). *Scottish Agriculture*, **34**, 68.

[*Received for publication, April 12th,* 1955]

THE ABSORPTION OF MAGNESIUM FROM THE ALIMENTARY TRACT OF SHEEP

By

J. Stewart

Moredun Institute, Gilmerton, Edinburgh

and

*E. W. Moodie

Royal (Dick) School of Veterinary Studies, Edinburgh

INTRODUCTION

There is still much controversy regarding the cause of seasonal hypomagnesaemia and hypomagnesaemic tetany in both cattle and sheep, and a survey of the literature reveals that little or nothing is known regarding the absorption of magnesium from the alimentary tract of ruminants. The following experiments, therefore, were carried out as a preliminary to the study of the factors influencing magnesium absorption and to ascertain if possible the region of maximum absorption. Sheep were selected as the most useful animals for the operational procedure and manipulation. They were not allowed food for the twelve hours preceding operation and at the end of each experiment were killed whilst still under anaesthesia.

OPERATIONAL PROCEDURE

Anaesthesia was induced and maintained by the use of Pentobarbitone Sodium Solution (1 gr. per ml.) given intravenously.

The experimental animal was placed on a heated operating table and the abdominal cavity was opened by an incision made in the right flank through the skin, abdominal muscles and peritoneum from a point at the edge of the last lumbar transverse process to the region of the xiphoid cartilage.

Blood samples were taken from the jugular vein immediately preceding dosing and after completion of the operation to observe the changes in the mineral concentration of the venous blood during the course of the procedure. Prior to, and at intervals during, the operation samples were taken from the carotid artery to represent arterial blood. Blood was also taken from the following veins to represent drainage from the various parts of the alimentary tract—right ruminal to indicate drainage from the rumen, left gastric from the abomasum, gastro-duodenal and anterior pancreatico-duodenal from the duodenum, intestinal from the intestine, caecal branch of the ileo-caecal-colic from the caecum, colic trunk of the ileo-caecal-colic from the colon, and the left colic to represent drainage from the terminal colon.

In one series of experiments samples of the contents of the various parts of the alimentary tract were taken at the same time as the blood samples representing drainage from these parts. This was done by means of cannulae inserted at the time of operation. The cannulae used for the rumen and caecum had an external diameter of 3 cm. and an internal

*Now of Queensland Veterinary School, Yeerongpilly, Brisbane, Australia.

diameter of 1.25 cm. while those used for the small intestine and abomasum had an external diameter of 8 mm. and an internal diameter of 5 mm.

Both blood and alimentary tract contents were assayed for calcium, inorganic phosphorus and magnesium—calcium by the Kramer-Tisdall oxalate precipitation and permanganate titration method, magnesium by a modification of the Denis method, and phosphorus by the Fiske and Subarrow method.

RESULTS

Absorption of Magnesium Throughout the Alimentary Tract

In the present work it was assumed that, if the concentration of an element in the blood from the vein draining a part of the alimentary tract was significantly higher than that of the succeeding sample from the carotid artery, absorption had taken place from that part. Objection to this assumption might be raised if there was a long interval between the taking of the venous and arterial samples, but in the present work the interval was very brief, being only of a few minutes' duration. The value for venous blood was always compared with the value of the succeeding carotid sample. Great care is necessary in the interpretation of the data and the general trend of values in the contents of both the venous and carotid samples must be examined as well as the values for the contents of the various parts of the alimentary tract under consideration. This method has been adopted by most of the workers in this field of ruminant physiology (Masson and Phillipson, 1951; Parathasarathy, Garton and Phillipson, 1952; Schambye, 1951; Scarisbrick and Ewer, 1951).

In order to ascertain whether the anaesthetic would have an effect on the blood minerals under examination two sheep, A and B, were anaesthetised and kept under total anaesthesia for four hours, samples being taken at intervals from the jugular vein. Two sheep, C and D, were kept in the same pen but not anaesthetised and were bled at similar intervals to A and B. It will be seen from Table 1 that anaesthesia had very little effect on the blood calcium, magnesium and inorganic phosphate, a greater variation being apparent in C and D than in A and B.

In the case of sheep 1, 2 and 3 the operational procedure was carried out to ascertain if the manipulation of the animals, consequent on the technique employed, would exert a greater effect on the blood minerals than that of total anaesthesia, as shown in A and B. From Table 1 it was seen that the procedure had little effect on either blood calcium or magnesium, although there was a slight variation in the contents of the various veins sampled. The variation in the phosphate content of the veins was greater than that of the calcium and magnesium, but there was no evidence of any serious alteration due to tissue response after manipulation. Much has been written regarding the diurnal variation of blood phosphate but the data in Table 1 showed little change over three or four hours.

From the data of Table 1 it was also apparent that little could be deduced regarding the site of magnesium absorption. There was

TABLE 1

EFFECT OF THE ANAESTHETIC AND OF THE OPERATIONAL PROCEDURE ON THE BLOOD MINERALS

Time	*Anaesthetised*						*No anaesthetic*					
	Sheep A mg/100 ml			*Sheep B mg/100 ml*			*Sheep C mg/100 ml*			*Sheep D mg/100 ml*		
	Ca	*Mg*	*P*	*Ca*	*Mg*	*P*	*Ca*	*Mg*	*P*	*Ca*	*Mg*	*P*
Pre	8·5	3·38	6·54	10·1	2·40	6·10	11·2	2·44	8·42	10·9	2·38	10·36
½ hr	8·5	3·38	5·74	10·1	2·41	6·23	10·9	2·44	9·26	10·9	2·78	9·80
1 ,,	8·5	3·35	5·54	10·1	2·42	6·41	10·9	2·44	7·56	10·9	2·40	9·26
2 ,,	8·5	3·31	5·90	10·0	2·40	6·28	10·9	2·48	7·40	10·9	2·36	9·52
3 ,,	8·5	3·29	5·74	10·2	2·40	6·30	10·9	2·42	7·26	11·2	2·27	9·80
4 ,,	8·3	2·81	5·54	10·1	2·38	6·10	10·9	2·12	6·76	11·2	2·03	8·70

Blood sample	*Sheep 1 mg/100 ml serum*			*Sheep 2 mg/100 ml serum*			*Sheep 3 mg/100 ml serum*		
	Ca	*Mg*	*P*	*Ca*	*Mg*	*P*	*Ca*	*Mg*	*P*
Jugular 1 ..	9·6	1·89	4·04	9·1	2·34	4·50	11·3	3·03	3·88
Carotid 1 ..	10·1	1·89	5·62	9·3	2·24	5·26	10·1	3·21	3·84
Rumen ..	9·6	2·09	4·71	8·8	2·08	4·97	10·9	2·87	4·52
Abomasum ..	8·5	2·01	7·14	9·1	2·18	4·97	11·1	3·14	5·60
Duodenum ..	9·9	1·87	8·85	9·0	2·17	4·68	11·1	3·56	4·76
Intestine ..	8·5	1·86	5·40	9·1	2·21	5·60	10·4	3·29	4·62
Caecum ..	9·1	2·27	5·40	9·1	2·22	5·52	10·4	3·56	5·19
Colon ..	9·9	1·75	5·13	9·9	2·29	4·97	9·7	3·21	5·84
Terminal colon ..	8·1	2·01	7·25	8·8	2·24	5·88	11·3	3·80	6·02
Carotid 2 ..	—	—	—	9·3	2·22	5·52	10·6	3·14	—
Jugular 2 ..	—	—	—	9·1	2·30	5·40	11·2	3·17	6·15

a remarkable constancy in the mineral content of all veins for any one animal and practically all the values were within the normal range for sheep.

In order to increase the magnesium content along the whole tract 4 oz. (112 g.) of magnesium sulphate were given orally to three sheep, (4, 5 and 6) and two hours later operation was commenced. In the case of 6, samples of the contents of the various parts of the alimentary tract were taken by cannulae as described above, but owing to the extremely solid nature of the contents of the colon and terminal colon it was not possible to obtain samples from these two regions. The results are shown in Table 2.

Slight absorption of magnesium took place from both the rumen and abomasum of both sheep 4 and 6 and from the abomasum of sheep 5. This was true also of the duodenum and small intestine

except in 6. It was apparent from the assay of the samples of the contents of the various parts of the tract that within the period

TABLE 2

ABSORPTION FROM ALIMENTARY CANAL AFTER DOSING WITH 112 g. MAGNESIUM SULPHATE

Time of sampling	*Sample*	*Sheep 4*			*Sheep 5*			*Sheep 6*		
		mg. per cent			*mg. per cent*			*mg. per cent*		
		Ca	*Mg.*	*P.*	*Ca.*	*Mg.*	*P.*	*Ca.*	*Mg.*	*P.*
Pre-dosing	Jugular Blood	10·6	2·32	5·60	10·66	2·39	5·67	9·1	2·78	9·95
	Jugular Blood	10·4	3·10	4·97	10·26	3·66	5·50	9·2	3·90	8·60
2 hrs. after dosing	Carotid Blood	10·0	3·27	5·95	9·86	4·17	7·50	9·1	3·90	8·70
2–2½ hrs. after dosing	Rumen Blood	10·2	4·04	6·55	10·13	4.09	7·02	9·3	5·53	9·35
	Rumen Contents	—	—	—	—	—	—	27·0	145·0	50·5
	Abomasum Blood	9·7	4·04	7·50	10·00	4·51	7·08	8·5	5·66	10·1
	Abomasum Contents	—	—	—	—	—	—	30·0	129·0	35·2
2½ hrs. after	Carotid Blood	10·0	3·66	6·50	9·78	4·19	6·88	8·8	4·28	11·5
2½–3 hrs. after dosing	Duodenum Blood	9·6	5·20	7·70	10·43	5·42	—	8·5	4·39	9·80
	Duodenum Contents	—	—	—	—	—	—	44·0	143·0	19·6
	Intestine Blood	9·3	4·93	8·05	10·40	5·56	7·13	8·0	4·40	9·35
	Intestine Contents	—	—	—	—	—	—	44·0	151·0	17·7
3 hrs. after	Carotid Blood	9·9	4·06	7·70	9·73	5·10	6·88	7·9	4·86	10·10
3-4 hrs. after dosing	Caecum Blood	9·9	4·97	10·45	9·73	6·06	7·02	8·3	5·36	9·48
	Caecum Contents	—	—	—	—	—	—	93·0	277·0	—
	Colon Blood	9·1	4·30	10·45	10·26	5·56	6·92	7·9	5·25	9·24
	Terminal Colon Blood	9·2	4·97	—	9·60	5·42	6·84	8·0	5·22	—
4 hrs. after	Carotid Blood	10·1	8·33	11·20	9·86	6·41	8·40	8·3	5·22	9·35
	Jugular Blood	10·2	7·66	11·0	10·26	6·06	8·47	8·0	5·49	9·95

of the experiment, four hours, the dose of magnesium sulphate had passed to the caecum, but there was no real evidence of absorption from the caecum and colon of magnesium or of calcium and phosphate.

As regards interaction of magnesium with calcium and phosphorus a point of interest is that whereas there was a slight fall in the calcium content of the venous and arterial blood there was a considerable rise in the inorganic phosphate contents, except in 6 which had a high phosphate content before the dose was given.

In order to see the effect of the magnesium ion the experiment was repeated using magnesium nitrate instead of sulphate, and so sheep 7 and 8 were given 112 g. magnesium nitrate two and a half hours previous to the operational procedure. To ascertain the

interaction of increased phosphorus and increased calcium intake on the magnesium content of the blood sheep 9 and 10 were given 112 g. sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) and sheep 11 was given 112 g. calcium chloride ($CaCl_2.6H_2o$). The analytical results are shown in Table 3.

The most striking result from the dosage of magnesium nitrate was the much enhanced absorption of magnesium, as the blood magnesium values even by two and a half hours were approximately 300 per cent higher than previous to the dosing—a level of blood magnesium never before observed so quickly after oral dosing. Similar results were obtained from both sheep, and by four hours after dosing the blood magnesium had reached 15.4 mg. and 10.0 mg. per 100 ml. of serum for sheep 7 and 8 respectively. The importance of this result in magnesium therapy would appear to be very great, but many aspects must be examined before suggesting its use in either the cure or prevention of hypomagnesaemia or in other diseases where magnesium salts are prescribed. Unfortunately, probably due to the very quick absorption of the salt, little could be deduced as to the part of the alimentary tract in which the absorption had taken place, except that in the case of sheep 8 there was obvious absorption of magnesium from the rumen. In both sheep 7 and 8 the phosphate content of the blood increased.

The large dose of sodium dihydrogen phosphate given to sheep 9 and 10 increased the inorganic phosphate content of the blood to abnormal values, but the magnesium content was only slightly affected, falling from 3.26 to 2.58 mg. and 3.17 to 2.64 mg. for 9 and 10 respectively. The large dose of phosphate had a much greater effect on the blood calcium which fell in both sheep to an extremely low level.

The dose of 4 oz. calcium chloride to sheep 11 increased its blood calcium in two and a half hours to nearly twice its pre-dosing level, and absorption of calcium was obvious from the rumen and abomasum. Both the blood magnesium and blood phosphate were only slightly increased by the large dose of calcium chloride.

In all sheep, 9, 10 and 11, absorption of magnesium was apparent from the rumen.

Many research workers in the field of mineral metabolism have suggested that the metabolic pathways of calcium and magnesium are different, and the above results would tend to support this view for ruminants.

Absorption of Magnesium from the Various Parts of the Alimentary Tract

Since the results from the experimental technique described in the previous section were not conclusive, another series of experiments was undertaken in which absorption of magnesium from each part of the alimentary tract was studied in turn. For this purpose the tract was divided into three sections—rumen and abomasum; duodenum and small intestine; and caecum, colon and terminal colon.

TABLE 3

ABSORPTION OF MAGNESIUM FROM ALIMENTARY TRACT AFTER DOSING WITH VARIOUS SALTS

Time of sampling	*Blood sample*	*Sheep 7*			*Sheep 8*			*Sheep 9*			*Sheep 10*			*Sheep 11*		
		112 g. $Mg(NO_3)_2$			*112 g. $Mg(NO_3)_2$*			*112g. $NaH_2PO_4 2H_2O$*			*112g. $NaH_2PO_4.2H_2O$*			*112 g. $CaCl_2.6H_2O$*		
		Ca. mg.%	*Mg.* mg.%	*P.* mg.%	*Ca.* mg.%	*Mg.* mg.%	*P.* mg.%	*Ca.* mg.%	*Mg.* mg.%	*P.* mg.%	*Ca.* mg.%	*Mg.* mg.%	*P.* mg.%	*Ca.* mg.%	*Mg.* mg.%	*P.* mg.%
Before Dosing	Jugular 1	9·71	2·47	8·05	10·40	2·50	9·26	9·6	3·26	8·25	9·84	3·17	11·90	9·33	2·19	6·12
	Jugular 2	9·71	10·00	8·95	9·45	8·70	9·80	8·0	3·03	19·75	6·79	2·79	18·65	23·68	2·54	9·46
2½ hrs. after	Carotid 1	9·71	10·53	9·96	9·45	8·90	10·74	8·0	2·56	26·75	6·38	2·60	18·50	23·95	2·47	9·40
2½–3 hrs. after	Rumen	9·45	10·77	10·70	9·45	10·26	10·90	7·6	2·66	28·65	6·65	3·30	19·50	29·25	2·74	8·25
	Abomasum	9·18	10·97	11·20	7·05	8·42	16·30	7·6	2·23	44·75	6·38	2·81	20·00	25·39	2·47	9·32
3 hrs. after	Carotid 2	9·18	11·43	10·60	8·78	8·90	11·08	7·6	2·42	30·85	6·25	2·30	19·75	23·95	2·47	9·52
3–3½ hrs. after	Duodenum	9·05	11·43	10·80	8·78	9·30	11·08	7·2	2·34	32·50	6·25	2·38	22·00	23·95	2.33	9.66
	Intestine	9·05	11·77	11·0	8·78	9·53	11·08	7·6	2·30	30·35	5·99	2·33	23·50	23·14	2·30	9·80
3½ hrs. after	Carotid 3	8·92	12·32	11·5	8·78	9·87	11·46	7·2	2·32	31·65	5·85	2·32	21·25	22·74	2·23	9·32
3½–4 hrs. after	Caecum	9·05	12·70	11·9	8·65	9·20	11·20	6·8	2·50	30·60	5·85	2·96	23·15	23·40	2·55	10·16
	Colon	8·78	12·70	11·6	8·78	9·53	11·20	6·8	2·48	31·65	5·99	2·63	22·20	22·46	2·14	9·83
	Terminal Colon	8·65	14·30	—	8·00	9·42	10·98	6·53	2·33	30·60	5·32	2·64	21·65	20·47	2·33	9·26
4 hrs. after	Carotid 4	9·60	15·70	13·7	8·00	10·26	11·90	6·53	2·58	35·10	5·59	2·62	23·80	23·68	2·30	11·08
	Jugular 3	9·45	15·40	—	8·13	10·00	10·70	6·80	2·58	33·05	5·99	2·64	23·15	22·35	2·31	9·96

The same operational procedure was carried out but cannulae were inserted in the section under investigation and the dose of magnesium sulphate was inserted by the cannula after pre-samples had been taken from the jugular vein, carotid artery and veins draining the particular section. At definite intervals after dosing further samples were taken from the veins and carotid artery. For each part of the tract samples of the contents were also withdrawn, via cannulae, after each bleeding in the case of at least one sheep.

For the discussion of the results from this section the data have been tabulated to show more clearly the arterio-venous difference.

Rumen and abomasum. Sheep 12 and 13 were given 112 g. of magnesium sulphate into the rumen and sheep 14, 60 g. into the abomasum. In the case of 13 and 14 the contents of the rumen and abomasum respectively were regularly sampled. An attempt was made to mix the contents as thoroughly as possible by injecting the dose into as many parts of the section as possible through the

TABLE 4

ABSORPTION OF MAGNESIUM FROM RUMEN AND ABOMASUM

Concentration of Magnesium mg/100 ml.

Time after dosage (mins)	*Carotid artery*	*Rumen vein*	*Arterio-Venous difference*	*Rumen contents*	*Abomasum vein*	*Arterio-Venous difference*	*Abomasum contents*
Sheep 12 (112 g $MgSO_4$ per rumen)							
Predosage	1·82	1·99	+0·17	—	2·01	+0·19	—
0–20	2·28	3·23	+0·95	—	2·73	+0·45	—
20–40	2·28	3·62	+1·34	—	3·48	+1·20	—
40–60	2·84	2·92	+0·08	—	2·38	−0·46	—
60–90	2·69	2·97	+0·28	—	2·97	+0·28	—
90–120	2·98	3·15	+0·17	—	3·23	+0·25	—
120–180	3·30	3·77	+0·47	—	3·66	+0·36	—
Sheep 13 (112 g $MgSO_4$ per rumen)							
Predosage	2·63	2·78	+0·15	12	—	—	—
0–20	3·56	4·04	+0·48	1140	—	—	—
20–40	3·27	4·64	+1·37	974	—	—	—
40–60	4·14	4·64	+0·50	648	—	—	—
60–90	4·14	6·00	+1·86	785	—	—	—
90–120	4·98	6·06	+1·08	480	—	—	—
120–180	5·68	7·32	+1·64	177	—	—	—
Sheep 14 (60 g $MgSO_4$ per abomasum)							
Predosage	3·05	—	—	—	3·08	+0·03	16
0–20	4·13	—	—	—	4·40	+0·27	1078
20–40	4·13	—	—	—	6·16	+2·03	845
40–60	4·94	—	—	—	6·66	+1·72	888
60–90	6·28	—	—	—	6·84	+0·56	534
90–120	6·62	—	—	—	7·02	+0·40	469
120–180	6·50	—	—	—	7·12	+0·62	421

cannula, and the same care was taken in the subsequent sampling of the contents. The results are given in Table 4.

In both 12 and 13 the blood from the rumen vein had always a higher magnesium content than that of the immediately succeeding sample from the carotid artery, thus showing that absorption of magnesium had taken place from the rumen. From the analysis of the rumen contents it is observed that twenty minutes after dosing the concentration of magnesium was about 100 times greater than before the dose was given and that the concentration fell in three hours to ten times the pre-dosing concentration, yet at the end of three hours absorption was apparently taking place more quickly than immediately after dosing. This may have been due to the mucosa of the rumen being in more intimate contact with the magnesium sulphate as the experiment progressed, although the concentration of magnesium within the rumen had decreased.

From the results of both 12 and 14, where in the one case the dose was given into the rumen and in the other into the abomasum itself, there is evidence of absorption of magnesium taking place from the abomasum; especially is this true one hour after dosing. The dose increased the concentration of magnesium in the abomasum of 14 very considerably and, just as in the case of the rumen injection, absorption took place even after the concentration was reduced, presumably by passage further along the tract.

It is concluded from these experiments that magnesium can be absorbed from both the rumen and the abomasum when a large dose of magnesium sulphate reaches that section of the alimentary tract.

Duodenum and small intestine. A similar technique was used for the duodenum and small intestine, cannulae being fixed in both and a dose of magnesium sulphate inserted via the cannulae. With the first sheep the dose inserted in the duodenum was 112 g. magnesium sulphate, but the animal died 30 minutes later with the blood magnesium raised to 19.05 mg./100 ml. serum and the calcium content 9.6 mg./100 ml. Two further sheep, given 50 g. magnesium sulphate by intestinal cannula, died within 30 minutes and each had a blood magnesium of 20 mg./100 ml. In all cases the magnesium/calcium ratio in the serum was 2 : 1. Allcroft (1947) found that after 90 g. magnesium sulphate given intravenously to a goat had raised the blood magnesium to 20.5 the goat died, the magnesium/calcium ratio being 2.5:1. Another goat given 90 g. magnesium sulphate subcutaneously died after seven hours with blood magnesium raised to 20.7 mg. and the magnesium/calcium ratio again 2.5:1. In the present experiments death ensued when the magnesium/calcium ratio reached 2:1 which is in agreement with the experimental results of workers using non-ruminant animals.

Sheep 15 and 16 were given 20 g. magnesium sulphate by duodenal cannula. Sheep 15 was selected as it had an extremely

low blood calcium and high magnesium. Sheep 17 and 18 were given 5 g. magnesium sulphate, 17 by the duodenum and 18 by

TABLE 5

ABSORPTION OF MAGNESIUM FROM DUODENUM AND SMALL INTESTINE

Concentration of Magnesium mg/100 ml.

Time after dosage (mins)	*Carotid artery*	*Duodenum vein*	*Arterio-venous difference*	*Duodenum contents*	*Intestine vein*	*Arterio-venous difference*	*Intestine contents*
Sheep 15 (20 g $MgSO_4$ per duodenum)							
Predosage	4·14	4·17	+0·03	—	4·30	+0·16	—
0–30	6·65	8·61	+1·96	—	6·72	+0·07	—
30–60	6·81	7·48	+0·67	—	8·45	+1·64	—
60–120	6·81	7·17	+0·35	—	6·84	+0·03	—
120–180	7·35	7·48	+0·13	—	8·79	+1·44	—
Sheep 16 (20 g $MgSO_4$ per duodenum)							
Predosage	2·79	2·95	+0·16	—	2·54	−0·24	—
0–30	4·09	8·15	+4·06	—	3·92	−0·17	—
30–60	3·82	5·29	+1·47	—	3·98	+0·16	—
60–120	4·09	3·82	−0·17	—	3·92	−0·17	—
120–180	3·34	4·23	+0·89	—	4·25	+0·91	—
180–240	3·25	4·70	+1·45	—	5·51	+2·26	—
Sheep 17 (5 g $MgSO_4$ per duodenum)							
Predosage	3·25	3·10	−0·15	5	—	—	—
0–30	3·25	3·38	+0·13	212	—	—	—
30–60	3·73	3·60	−0·13	221	—	—	—
60–120	4·23	4·06	−0·17	64	—	—	—
120–180	4·87	4·32	−0·55	25	—	—	—
180–240	4·72	5·00	+0·28	18	—	—	—
Sheep 18 (5 g $MgSO_4$ per small intestine)							
Predosage	3·75	—	—	—	3·05	−0·70	24
0–30	3·84	—	—	—	4·55	+0·71	696
30–60	4·36	—	—	—	4·72	+0·36	298
60–120	4·12	—	—	—	4·74	+0·62	258
120–180	4·12	—	—	—	4·15	+0·03	283
180–240	4·17	—	—	—	4·17	—	206

the small intestine. The usual operational procedure was carried out in each animal and the results are shown in Table 5.

In the case of both 15 and 16 there was obvious absorption from the duodenum within 30 minutes of dosing, the magnesium content of the blood of the duodenal veins being 8.61 and 8.15 mg. with an arterio-venous difference of +1.96 and +4.06 respectively. As the salt passed from the duodenum to the small intestine it was very clearly shown in both sheep that absorption from the duodenum was reduced and absorption from the small intestine was increased,

until at the end of the period the magnesium contents of the blood from the small intestine veins were 8.79 and 5.51 mg. as compared with 7.48 and 4.70 mg. for the duodenum vein. Absorption of magnesium was not so clearly demonstrated in the case of sheep 17 and 18 after the smaller doses of 5 g. magnesium sulphate, although the concentration of magnesium in both duodenum and small intestine was about 50 times the pre-dosing concentration.

Caecum and colon. In one of the animals only (sheep 3) mentioned in the first section the values for the veins draining the caecum, colon and terminal colon (Table 1), had suggested that absorption might be occurring in that area of the alimentary tract, but it was decided to obtain more data. 50 g. of magnesium sulphate were given to 19, and 20 g. to 20 by means of a caecal cannula. From 20 caecal contents were taken as well as blood samples. Difficulties

TABLE 6

ABSORPTION OF MAGNESIUM FROM CAECUM

Concentration of Magnesium mg/100 ml.

Time after dosage (mins)	*Carotid artery*	*Caecum vein*	*Arterio-venous difference*	*Caecum contents*	*Colon blood*	*Arterio-venous difference*	*Terminal colon blood*	*Arterio-venous difference*
Sheep 19 (50 g $MgSO_4$ per caecum)								
Predosage	2·47	2·27	−0·20	—	2·22	−0·27	2·49	+0·02
0–30	2·47	2·60	+0·13	—	2·48	+0·01	2·49	+0·02
30–60	2·78	2·67	−0·11	—	2·48	−0·30	2·65	−0·13
60–90	3·21	3·08	−0·23	—	3·00	−0·21	3·40	+0·19
90–120	3·25	3·30	+0·05	—	3·29	+0·04	3·33	+0·08
120–150	3·72	3·62	−0·10	—	3·84	+0·12	3·61	−0·11
Sheep 20 (20 g $MgSO_4$ per caecum)								
Predosage	2·76	2·84	+0·08	53	—	—	—	—
0–30	4·35	19·66	+15·11	580	—	—	—	—
30–60	8·05	17·61	+9·55	763	—	—	—	—
60–90	7·18	10·50	+3·32	777	—	—	—	—
90–120	6·67	6·91	+0·24	600	—	—	—	—
120–150	6·72	6·91	+0·19	464	—	—	—	—
150–180	6·40	6·57	+0·17	410	—	—	—	—

in sampling the colon owing to the solid nature of the contents prevented the injecting of salts into that part or into the terminal colon. The results from 19 and 20 are given in Table 6.

The increased content of magnesium in the final blood samples showed that absorption of magnesium must have taken place from the caecum or the colon since there was no chance of the salt percolating back to the small intestine, but in the case of 19 the blood from caecal, colon and terminal colon veins had a slightly higher content of magnesium than that of the carotid artery in only one or two instances. On the other hand 20 showed very

rapid and very large absorption from the caecum. By 30 minutes after dosing the caecal vein sample had a magnesium content of 19.46 mg. with an arterio-venous difference of +15.11. Throughout the three hours of the experiment an exceedingly high magnesium content was maintained in the caecal blood. The marked difference in the values for 19 and 20 is difficult to understand unless in the case of 20 the dose, though less in quantity, may have been added to a much less total volume of caecal contents or may have been placed in much closer contact with the mucous membrane.

As was evident from the results of the first section every animal receiving a dose of magnesium sulphate had a much higher blood phosphate at the end of the experiment than at the beginning, irrespective of where in the alimentary tract the salt had been added.

Comparison of Table 7 with Table 1 shows that in most cases the rise in blood phosphate was much higher after magnesium dosage than after total anaesthesia or simple operation without additional

TABLE 7

ALTERATION IN CALCIUM AND PHOSPHORUS CONTENT OF BLOOD IN JUGULAR VEIN DURING COURSE OF EXPERIMENT

mg/100 ml.

Sheep No.	*Dose*	*Calcium mg/100 ml*		*Inorganic phosphorus mg/100 ml*	
		Before dosing	*End of experiment*	*Before dosing*	*End of experiment*
12.	112 g. $MgSO_4$ per rumen ..	10·78	9·18	7·80	8·96
13.	112 g. $MgSO_4$ per rumen ..	10·70	9·70	6·29	10·60
14.	60 g. $MgSO_4$ per abomasum	9·33	9·30	5·94	11·80
15.	20 g. $MgSO_4$ per duodenum	5·45	4·78	9·96	14·04
16.	20 g. $MgSO_4$ per duodenum	9·60	10·90	8·60	15·10
17.	5 g. $MgSO_4$ per duodenum ..	10·10	9·30	5·94	10·92
18.	5 g. $MgSO_4$ per small intestine	9·90	9·30	8·28	13·40
19.	50 g. $MgSO_4$ per caecum ..	10·70	9·60	7·80	11·30
20.	20 g. $MgSO_4$ per caecum ..	9·10	9·20	7·36	11·20

magnesium being given. In no case, however, was it possible to ascertain if absorption of phosphate had taken place from any specific part of the alimentary tract. Moreover it was confirmed that the increases in the magnesium and phosphate contents of the blood were not proportional.

From the present series of experiments it was impossible to determine whether the large doses of magnesium sulphate had directly influenced phosphate absorption from the alimentary tract or whether the large increases in phosphate content of the blood as the operation proceeded were exaggerated effects of the operational procedure although the values in Table 1 would tend to show that the manipulation of the tissues during operation did not seriously affect the blood phosphate level. Table 7 also shows that

the calcium content of the blood tended in most cases to fall slightly. This fall could not be correlated with the very substantial rises in magnesium content of the blood after magnesium sulphate had been given.

Thus from the above two series of experiments it is concluded that under certain circumstances magnesium may be absorbed from the rumen, abomasum, duodenum, small intestine and caecum, and apparently in largest amounts from the duodenum and small intestine.

CONCLUSIONS

Experiments have been carried out on anaesthetised sheep to discover in what parts of the alimentary tract absorption of magnesium takes place. Venous blood from the right ruminal, left gastric, gastro-duodenal and anterior pancreatic-duodenal, intestinal, caecal and colic branches of the ileo-caeco-colic and left colic veins was taken by syringe at different intervals after the introduction of magnesium salts by mouth or through cannulae. These blood samples, which were considered to be representative of drainage from the various parts of the alimentary tract, were checked against arterial blood taken from the carotid artery at comparable intervals.

It was shown that after heavy doses of magnesium sulphate under certain circumstances, absorption of magnesium may take place from the rumen, abomasum, duodenum, small intestine and caecum. The principal site of magnesium absorption is probably the duodenum and small intestine.

After large doses of magnesium salts the phosphate content of the blood was also increased but the calcium content was slightly decreased. There was no evidence to show that calcium and magnesium followed a similar pathway of metabolism.

It was also shown that magnesium was much more quickly and efficiently absorbed after doses of magnesium nitrate by mouth than after similar amounts of magnesium sulphate. The importance of this in veterinary medicine has yet to be studied.

REFERENCES

Allcroft, W. M. (1947). *Vet. J.*, **103**, 157.
Masson, M., and Phillipson, A. T. (1951). *J. Physiol.*, **113**, 189.
Parathasarathy, D., Garton, G. A., and Phillipson, A. T. (1952). *Biochem, J.*, **52**, XVI.
Scarisbrick, R., and Ewer, T. K. (1951). *Ibid.*, **49**, LXXIX.
Schambye, P. (1951). *Nord. vet. Med.*, **3**, 555.

[*Received for publication, April 12th,* 1955]

FURAZOLIDONE IN THE CONTROL OF HISTOMONIASIS (BLACKHEAD) IN TURKEYS

By

C. Horton-Smith and P. L. Long

Poultry Research Station, Animal Health Trust, Houghton, Huntingdon

INTRODUCTION

There is evidence that birds, treated with appropriate chemical compounds for histomoniasis with apparent success, may again develop symptoms of the disease when treatment stops. Relapse, and not necessarily re-infection, may be responsible for this since the drug used may merely inhibit increase of the histomonads which will resume multiplication when medication is withdrawn. In view of this, there is obviously a need for some form of preventive medication that will overcome any recurrence of the disease.

McGregor (1953), describing preliminary observations on a series of nitrofuran compounds including N—(5-nitro-2 furfurylidene)—3-amino-2-oxazolidone or furazolidone (also referred to as N.F. 180), stated that this compound showed a slight prophylactic effect against histomoniasis when used continuously in the food at a concentration of 1 : 8000 from two days before initial infection with *Heterakis* ova. His data show that deaths from histomoniasis amounted to 76·47 per cent in the treated, and 100 per cent in the untreated groups. When he increased the concentration of the drug to 1 : 4000 and offered it to turkeys immediately after blackhead symptoms appeared the mortality was 88·24 per cent. He pointed out that more work with this compound might be justified. Harwood and Stunz (1954) reported that three-week-old poults got some protection when offered furazolidone in the food from the second, ninth and sixteenth days after receiving *Heterakis* ova and that the toxicity of the drug is low, the LD_{50} for chickens being about 500 mg. per kilo.

Although a study of McGregor's results showed a high mortality in his treated groups, it was decided to investigate the possible use of the drug in the therapy of histomoniasis. We embarked on the tests with little confidence that furazolidone would give results at all comparable with those obtained with 2-amino-5-nitrothiazole (Horton-Smith and Long, 1951).

MATERIALS AND METHODS

At the commencement of experiments 1, 2, 3, 5 and 6 the poults used in them were six weeks old or less, were reared indoors on wire floors and, when needed for experimental purposes, were transferred to solid floor pens with sawdust litter, heat being supplied by dull emitter infra-red lamps. The poults used in experiment 6 were removed from the solid floor pen to a wire-floor fold unit, when treatment was completed. The poults used in experiment 7 were reared out of doors in wire floor

brooders heated by paraffin lamps and were, when the experiment started, seven weeks old and then removed to slatted floor arks with wire runs. In experiments 4 and 8 turkeys twelve weeks old or more were used; they were reared similarly to those used in experiment 7 and were later, removed to fold units with slatted floors. To ascertain if the breed or age of the bird would influence the results, Broad Breasted Bronze and British White turkeys of mixed sexes and different ages were used, the oldest being eighteen weeks at initial infection.

Food. At the beginning and throughout the duration of the experiment, poults six weeks old or less received the following ration: ground wheat 20, ground barley 20, Sussex ground oats 10, maize meal 11¾, middlings 15, white fish meal 5, decorticated groundnut meal 5, died grass meal 5, dried skim milk 5, unextracted dried yeast 2, minerals 1¼. This mash was supplemented with synthetic vitamin A 2000 i.u./lb., vitamin D_3 450 i.u./lb., and vitamin B_2 1 mg./lb. Poults over six weeks old at the start of an experiment received for the whole of the test period the following ration: ground barley 24, ground oats 20, maize meal 16¾, middlings 25, white fish meal 3, decorticated groundnut meal 5, dried grass meal 5, minerals 1¼. This mash was supplemented with synthetic vitamin A 2000 i.u./lb., and vitamin D3 450 i.u./lb. No other food was offered to the turkeys under experiment.

Preparation of medicated food. The furazolidone was first ground in with plain wheat flour to form a smooth 10 per cent premix completely free from small aggregations of the drug. For all the experiments, except No. 8, the medicated food was prepared by adding the premix to the requisite amount of food and mechanically mixing this in an electrically-driven, stainless-steel "Hobart" mixer. It was found convenient to mix 15 lb. of food for at least 15 minutes. The 2-amino-5-nitrothiazole was incorporated in the food in the same way except that the manufacturer's premix (22.5 per cent) was used. In experiment No. 8, larger quantities of food were needed as the turkeys were 16 to 18 weeks old and the food consumption per bird per week averaged 3 to 4 lb. over the experimental period. In this instance, 30 to 40 lb. of medicated food was mixed for 15 minutes at four-day intervals in a "worm" type mixer made of cast iron with the interior painted.

Infections. The turkeys were infected by introducing by pipette into the crop of each bird single doses of 500 to 1,000 embryonated *Heterakis* ova derived from mature worms collected from the caeca of domestic fowls. The criteria used to assess the value of medication were the differences in mortality from histomoniasis between the medicated and non-medicated groups of birds and the presence of lesions in the caeca and livers of turkeys sacrificed at the end of the experiment. In two experiments a second dose of embryonated *Heterakis* ova was given during treatment to find out if a subsequent heavy infestation would be a serious challenge to medication.

RESULTS

The Effect of Commencing a Course of Continuous Medication before Administering Embryonated Heterakis *Ova.*

We approached this investigation from the standpoint of prevention because of the nature of the disease and our experience with other compounds.

Experiment 1. Twenty four-week-old B.W. turkey poults were divided into two groups of ten birds each, one group being given food containing 0·02 per cent w/w furazolidone for 28 days. Two days after medication started each poult was infected with 500 *Heterakis* ova. The other group of birds was given non-medicated food and each poult was given 500 ova at the same time as those of the first group.

In the medicated group no deaths from histomoniasis occurred whereas in the non-medicated group there were five deaths ; one occurred on the 17th, one on the 18th, two on the 21st and one on the 23rd days after administration of the *Heterakis* ova. Two poults in the medicated and one in the control group died as a result of bullying. The surviving poults, four in the control and eight in the medicated group, were killed four days after the last non-medicated poult died, which was one day after medication stopped. At autopsy two of the control birds had advanced caecal infections, a third had haemorrhagic patches in the caecal wall, and the fourth was normal; all were in fair condition. In the medicated group, two poults showed caecal lesions, one with haemorrhage: the remaining six birds were in good condition with normal caeca and livers.

Although no deaths from histomoniasis occurred in the medicated group, the presence of lesions in two poults suggested that more serious infections might have developed had the birds been allowed to live longer after medication ceased.

Experiment 2. Since no deaths from histomoniasis occurred in the treated group in the previous experiment, it was decided to test the inhibitory effect on the mortality of two lower concentrations of the drug as well. Three medicated feeds containing furazolidone at the levels of 0·02, 0·01 and 0·0075 per cent w/w respectively were fed to each of three groups of four-week-old B.B.B. turkey poults. Eight birds were in the group given the 0·02 per cent medicated feed while

TABLE 1

EFFECT OF FEEDING DIFFERENT FURAZOLIDONE CONCENTRATIONS ON THE DEVELOPMENT OF HISTOMONIASIS WHEN GIVEN BEFORE *HETERAKIS* INFECTIONS

Group	*No. of birds*	*Per cent drug in food*	*Deaths from Histomoniasis*							*Total*
			Days after Heterakis *infection*							
			10	*13*	*14*	*15*	*16*	*17*	*19*	
1	8	0·02								0
2	10	0·01							a	1
3	10	0·0075								0
Control	10	nil	a	b	bb	bb	bb	bb		10

a = caecal lesions alone ; b = both caecal and liver lesions.

10 birds in each group were given the two other concentrations of the drug. A fourth, control group of 10 birds received non-medicated food. The experimental poults had access to the different medication feeds for 30 days, which was the period of the experiment, and all birds received 500 embryonated *Heterakis* ova two days after medicated feeding had started. The results are shown in Table 1. Only one bird given furazolidone died. All ten control birds died within 17 days. The surviving poults were killed twenty-eight days after receiving the *Heterakis* ova and their caeca and livers examined.

Caeca. No birds given the drug at 0·02 and 0·01 per cent in the food showed severe lesions although four turkeys receiving the former concentration and six birds receiving the latter showed thickening of the caecal walls with some necrotic material in the caeca. In the group receiving the 0·0075 per cent concentration of the drug, severe infections with necrotic cores in, and conspicuous enlargement of, the caeca occurred in two poults, the remaining birds having only a few ill-defined lesions. *Heterakis* worms were also recovered from the caeca of two turkeys in this group.

Livers. No turkeys given the drug at 0·02 per cent concentration showed macroscopical lesions, one bird in the 0·01 per cent group showed a single small lesion and six in the 0·0075 per cent group had several lesions.

Experiment 3. Two groups of four-week-old B.B.B. turkeys were used. The experimental group of 19 birds was given food containing 0·01 per cent w/w furazolidone, while the control group of 14 birds, was given similar, but non-medicated, food. Each poult in both

TABLE 2

EFFECT OF FEEDING 0.01 PER CENT FURAZOLIDONE ON THE DEVELOPMENT OF HISTOMONIASIS WHEN GIVEN BEFORE *HETERAKIS* INFECTIONS

Group	*No. of birds*	*Per cent drug in food*	*Deaths from Histomoniasis*					*Total*
			Days after Heterakis *infection*					
			12	*14*	*15*	*17*	*19*	
Expt.	19	0·01				a	a	2
Control	14	nil	b	bb	bb bb	bb b	b	11

a=caecal lesions alone ; b=both caecal and liver lesions.

groups received 580 embryonated *Heterakis* ova two days after medicated feeding was commenced. The results are shown in Table 2. All surviving poults were killed twenty six days after the administration of the *Heterakis* ova.

Caeca. In the medicated group, nine of the seventeen survivors showed thickening of the caecal wall and all three surviving control birds showed lesions.

Liver. Three birds in the medicated group showed a few ill-defined lesions and all three surviving control birds showed lesions.

Experiment 4. Because two experimental poults in Experiment 3 had shown gross caecal lesions at death whilst receiving food containing 0·01 per cent furazolidone, it was decided to increase the amount of the drug to 0·015 per cent. Food, containing the drug at this concentration, was given to eighteen twelve weeks old B.W. turkeys, for eight weeks commencing on the day preceding the administration of 500 embryonated *Heterakis* ova to each. A control group of eighteen turkeys, similar in age and breed, received *Heterakis* ova and non-medicated food. A second quantity of 500 ova was given to each poult of both groups thirty nine days after the first

TABLE 3

EFFECT OF FEEDING 0.015 PER CENT FURAZOLIDONE ON THE DEVELOPMENT OF HISTOMONIASIS WHEN GIVEN BEFORE *HETERAKIS* INFECTIONS

Group	No. of birds	Per cent drug in food	Deaths from histomoniasis									
			Days after Heterakis infection									
			11	12	15	17	18	39	55	58	70	Total
Expt.	18	0·015						Second dose of *Heterakis ova*	End of medication		All survivors killed and examined	0
Control	18	nil	b	b	b	b	b			b		13
					b	b	b					
					b	b	b					
							b					

b = both caecal and liver lesions present.

dose. The results are shown in Table 3. All surviving birds were killed fifteen days after medication had ceased, i.e., 70 days after receiving the first dose of *Heterakis* ova.

Caeca. Typical blackhead lesions were seen in five of the 18 birds in the medicated group and none in the five turkeys of the control group which survived. *Heterakis* worms were present in all five control and in ten of the medicated birds.

Livers. In the medicated group six turkeys, including those that had caecal lesions, showed liver lesions whereas none of the control birds was affected.

The Effect of Commencing a Course of Continuous Medication at Varying Times after Administering Embryonated Heterakis *Ova*

Two experiments were made to find the efficiency of furazolidone in controlling a developing histomonad infection.

Experiment 5. Twentyfour three-week-old B.B.B. poults were fed 500 embryonated *Heterakis* ova and were divided into three groups of eight birds. Group 1 was offered food containing 0·03 per cent w/w furazolidone for 14 days starting on the third day after infection. Group 2 was similarly treated except that the medicated food was first given 10 days after infection. The controls (Group 3,) were given non-medicated food throughout the experiment. The results are shown in Table 4. Although there was some reduction and delay in mortality among the treated birds, it appeared that the effect of

TABLE 4

EFFECT OF FEEDING 0.03 PER CENT FURAZOLIDONE AFTER *HETERAKIS* INFECTIONS ON THE MORTALITY DUE TO HISTOMONIASIS

Group	*No. of birds*	*Per cent drug in food*	*Days between infection and start of treatment*	*Deaths from histomoniasis*											*Total*
				Days after Heterakis *infection*											
				15	*16*	*17*	*18*	*24*	*27*	*28*	*31*	*35*	*36*	*37*	
1	8	0·03	3			S			b	b					2
2	8	0·03	10					S			b	b	b	b b	5
Control	8	nil	—	b	b	b b	b b b								7

S= end of treatment ; b=both caecal and liver lesions.

furazolidone was only inhibitory because deaths from histomoniasis occurred within seven to ten days after treatment ceased. All the surviving poults were killed and examined 79 days after infection and none showed any caecal or liver lesions.

Experiment 6. This experiment was designed to test the efficiency of furazolidone, fed at two concentrations, in controlling histomoniasis after an initial, followed by a second, dose of *Heterakis* ova.Thirty-seven six-week-old B.B.B. poults were divided into 4 groups containing 10, 10, 10 and 7 birds respectively. Birds in Group 3 acted as controls for the initial worm egg infections and those in Group 4 were controls for the re-infections. When the experiment started each poult of Groups 1, 2 and 3 was fed 500 *Heterakis* ova; those of Group 4 received none. Two days after this infection, Group 1 received food containing 0·005 per cent and Group 2 food containing 0.01 per cent of the drug; those of Groups 3 and 4 received non-medicated food. The treatment continued for 56 days. Twenty-four days after the initial *Heterakis* infection, Groups 1, 2 and 4 each received 800 worm ova. Thus the total ova fed to Groups 1, 2, 3 and 4 were 1,300, 1,300, 500 and 800 respectively. The results of this experiment are shown in

TABLE 5

EFFECT OF FEEDING 0.005 AND 0.01 PER CENT FURAZOLIDONE, AFTER AN INITIAL AND A SECOND *HETERAKIS* INFECTION, ON THE MORTALITY DUE TO HISTOMONIASIS

Group	*No. of birds*	*Per cent drug in food*	*Deaths from histomoniasis*																
			Days after receiving 500 Heterakis *ova*									*Days after receiving 800* Heterakis *ova*							*Total*
			13	*14*	*15*	*16*	*17*	*18*	*20*	*24*	*27*	*17 (41)*	*18 (42)*	*19 (43)*	*25 (49)*	*26 (50)*	*27–40 (51–64)*	*41 (65)*	
1	10	0·005				b		b	b b	H	a		b				S		6
2	10	0·01								H							S	b	1
3	10	nil	b	b	b	b b	b	b		—					b	b	—		9
4	7	nil	*No* Heterakis *ova given*							H		b b b	b b b	b			—		7

The figures in brackets refer to the number of days after the initial *Heterakis* infection ; a=caecal lesions alone ; b=both caecal and liver lesions ; H=each bird received 800 *Heterakis* ova ; S=medication stopped on the 58th day after first *Heterakis* infection.

Table 5. On the day on which the medicated food was completely withdrawn the surviving turkeys were transferred to a single wire floor fold unit and fourteen days later were killed and their livers and caeca examined. None of the birds showed evidence of liver infection but all showed some infection of the caeca except one from Group 1 and one from Group 2. These caecal infections were slight and caseous plugs were seen in only two instances; the tips of the caeca were chiefly involved and showed slight haemorrhages and thickening. Although it is not possible with certainty to associate deaths in groups 1, 2 and 3 with the second infection with *Heterakis* ova, it can be assumed within the time limit of the experiment that *Histomonas* establishments induced by these infections were largely suppressed in the treated group 2 as compared with those in the two sets of controls (groups 3 and 4).

This experiment indicated that furazolidone at 0·01 per cent w/w in the food afforded marked protection against the development of histomoniasis when the start of medication was delayed until the second day after dosing with *Heterakis* ova. There was little protection when the drug was given at 0·005 per cent.

A Comparison of the Effects of Commencing Courses of Continuous Medication with (a) furazolidone and (b) 2-amino-5-nitrothiazole before the Administration of Embryonated Heterakis *Ova.*

Experiment 7. Thirtyeight B.W. turkeys, seven weeks old, were divided into 3 Groups comprising 13, 12 and 13 birds respectively. Group 1 received 2-amino-5-nitrothiazole at a concentration of 0·05 per cent w/w in the food while Group 2 received furazolidone at a concentration of 0·015 per cent w/w in the food. Group 3, was the control. Each bird of all groups was given 500 embryonated *Heterakis* ova twenty-four hours after the medicated foods were first offered to Groups 1 and 2. The two medicated rations were given continuously for thirtyfive days, that is, until the thirty fourth day after the poults were dosed with worm eggs. The results of this experiment are shown in Table 6. The five surviving control birds of Group 3 and six birds from each of Groups 1 and 2 were killed and examined two days after medication had stopped. None showed caecal or liver lesions but *Heterakis* worms were recovered from the caeca of one of the Group 1, from four of the Group 2, and from three of the control birds. Eight days after medication had stopped all the remaining birds were killed and examined. None of the six surviving birds of Group 1 showed lesions although eight *Heterakis* worms were recovered from their pooled caecal contents. While no lesions were seen in the livers of the five surviving poults of Group 2, the caeca of two birds showed typical lesions and *Heterakis* worms were recovered from the caecal contents of three birds.

TABLE 6

COMPARISON OF THE EFFECTS OF FEEDING 2-AMINO-5-NITROTHIAZOLE AND FURAZOLIDONE ON THE DEVELOPMENT OF HISTOMONIASIS WHEN GIVEN TWENTY-FOUR HOURS BEFORE *HETERAKIS* INFECTIONS

Group	*No. of birds*	*Per cent and kind of drug in food*	*Deaths from histomoniasis*							*Total*
			Days after Heterakis *infection*							
			16	*17*	*18*	*19*	*26*	*27–40*	*41*	
1	3	0·05 2-amino-5-nitro-thiazole						Medication stopped on 34th day	1 poult killed by bullying	0
2	12	0·015 furazo-lidone							a	1
3	13	nil	b	b	b	bb	b			8
Control						b	b			

a = caecal lesions alone ; b = both caecal and liver lesions.

A Comparison of the Effects of Commencing Courses of Continuous Medication with (a) furazolidone and (b) 2-amino-5-nitrothiazole after the Administration of Embryonated Heterakis *Ova.*

Experiment 8. Sixty turkeys were divided into four groups each comprising nine 18 week-old B.B.B. and six 16 week-old B.W. birds. Each turkey was given 1,000 embryonated *Heterakis* ova. Group 1 received food containing 0·04 per cent w/w furazolidone while Groups 2 and 3 received food containing 0·04 per cent and 0·1 per cent respectively w/w 2-amino-5-nitrothiazole. Group 4 acted as controls. Medicated feeding of Groups 1, 2 and 3 started ten days after the *Heterakis* infections and was continued for fourteen days. One B.B.B. turkey of Group 3 was found dead as a result of bullying and two B.W. turkeys, one from each of Groups 1 and 2, were badly injured as a result of bullying and were killed. When these birds were examined post-mortem no caecal or liver lesions were detected although *Heterakis* worms were recovered from the caecal contents. The results of this experiment are shown in Table 7. The surviving turkeys were killed fortynine days after being dosed with *Heterakis* ova, or twenty-five days after medication had stopped, and none showed typical liver or caecal lesions at examination post-mortem. The pooled caecal contents of the birds of each group were examined for *Heterakis* worms and 88 specimens were recovered from Group 1 (0·04 per cent furazolidone), 23 from Group 2 (0·04 per cent 2-amino-5-nitrothiazole), 4 from Group 3 (0·1 per cent 2-amino-5-nitrothiazole), and 33 from Group 4 (controls). A packing station which accepted the forty-two turkeys on the medicated foods and returned the viscera to us for examination was entirely satisfied with the condition and weight of these birds for marketing purposes.

TABLE 7

COMPARISON OF THE EFFECTS ON THE DEVELOPMENT OF HISTOMONIASIS OF FEEDING FURAZOLIDONE AND 2-AMINO-5 NITROTHIAZOLE AFTER *HETERAKIS* INFECTIONS

Group	*No. of birds*	*Per cent and kind of drug in food*	*Deaths from histomoniasis*									
			Days after Heterakis *infection*									*Total*
			11	*18*	*19*	*20*	*21*	*23*	*24*	*26*	*27*	
1	15	0·04 furazolidone							Medication stopped		D	0
2	15	0·04 2-amino-5-nitrothiazole									D	0
3	15	0·1 2-amino-5-nitrothiazole	D									0
Control	15	nil		b b	b	b	b	b b		b		8

D=died or killed as a result of bullying ; b=both caecal and liver lesions.

DISCUSSION

Furazolidone mixed in the food in concentrations of 0·0075—0·02 per cent w/w, when offered to turkeys before the administration of *Heterakis* ova, prevented or markedly reduced the mortality arising from experimentally induced histomoniasis. When turkeys were fed on a medicated ration containing 0·0075 per cent w/w furazolidone before infection, examination post-mortem of the turkeys sacrificed during treatment showed that lesions were present in the caeca and the livers. These findings indicated that the compound at this concentration only partially suppressed the earlier caecal phases of the histomonad cycle. Turkeys which had been offered 0.02 per cent furazolidone for twenty eight or thirty days commencing two days before infection with *Heterakis,* and were sacrificed during treatment or twenty four hours after its cessation, showed only small caecal lesions (Experiments 1 and 2). Turkeys which received *Heterakis* infections on the first day following, and again on the thirty ninth day following the commencement of feeding with 0·015 per cent w/w furazolidone for fifty six days and sacrificed fifteen days after withdrawal of medication, showed lesions in the liver (Experiment 4). On the other hand, when turkeys had received the same treatment for thirty five days from the day before infection, but had only received one dose of *Heterakis* ova, no macroscopical lesions were found in those sacrificed two days after medication had ceased, but some

caecal lesions were noted in those sacrificed six days later (Experiment 7). In Experiment 6, in which treatment was delayed, substantial protection was afforded by a 56 day course of treatment with 0·01 per cent furazolidone in food offered to turkeys from the second day after infection and by a 0·04 per cent medicated feed offered from the tenth day of the infection and for fourteen days (Experiment 8). The turkeys in Experiment 6 were sacrificed on the fourteenth day after treatment had ceased and examination post-mortem showed slight caecal involvement in most of them. The turkeys in Experiment 8 were killed on the thirty ninth day after the cessation of treatment. No deaths had occurred in this experiment and there was no evidence of caecal or liver lesions at autopsy although the possibility cannot be ignored that macroscopic lesions might have developed if the birds had been retained alive for a longer period. There was marked delay in the time of mortality among turkeys receiving a fourteen day treatment with 0·03 per cent furazolidone from the third and tenth days after infection (Experiment 5). No lesions were noted in the caeca and livers of the survivors sacrificed at 55 or 62 days after treatment had ceased. The results of this experiment are difficult to explain other than on the grounds that the disease had cleared up spontaneously, as often happens in surviving control birds. In view of the mortality it is obvious that this concentration of the compound will not effectively hold an established infection. Provisionally, it is suggested that furazolidone at concentrations of 0·01 to 0·02 per cent w/w in the feed would be appropriate concentrations to adopt in continuous medication. Should furazolidone be considered for the treatment of established infections the concentration in the feed may have to be increased to 0·04 per cent w/w for at least fourteen days. This concentration of the drug appeared to effect a suppression of the infection in the single experiment performed (Experiment 8). Subsequent work has shown that relapse may follow cessation of treatment with 0.04 per cent. furazolidone and it is suggested that such treatment be followed by a course of medication at the preventive level.

No marked anthelmintic effect appears to be exerted by furazolidone. In this connection it is interesting to recall that Baker (1933) showed that few *Heterakis* worms developed to maturity in turkeys suffering from histomoniasis and that Wehr (1954) found that turkeys dying of the disease harboured large numbers. In our experiments adult worms were recovered from all groups although these numbers were small when compared with the numbers of ova administered.

At the concentrations used in our experiments there was little to choose between 2-amino-5-nitrothiazole and furazolidone when relative mortality from blackhead alone was considered (Experiments 7 and 8). A single experiment was undertaken to determine the effect of furazolidone (0·015 per cent in the feed) on an experimentally

induced intestinal coccidiosis of turkeys. The medicated food was offered to poults twenty-four hours before infection with a suspension of sporulated oocysts (suspected *Eimeria adenoeides*) and for the duration of the test. Eleven of the thirteen infected poults died and at examination heavy coccidial infections of the lower intestines were found.

Histomoniasis differs from coccidiosis in that no marked immunity appears to be stimulated by any definite phases in the life history of *Histomonas meleagridis*. Whereas an immunity to coccidiosis develops in the host in spite of suppression or destruction of schizogonous stages by medication, no such phenomenon appears to occur in histomoniasis. There is ample evidence to show that although symptoms of histomoniasis are suppressed during the period of medication the disease may once again become patent when treatment has ceased. This recurrence of disease has frequently been ascribed to reinfection when, in point of fact, it often arises from the continued multiplication of histomonads after treatment has stopped. Where medication was initiated before the *Heterakis* ova were administered very few or no deaths from histomoniasis occurred, but at examination post-mortem lesions were usually found in the caeca and in some cases in the livers as well. There were few or no lesions in the surviving control turkeys killed at the same time (Experiment 4) and the indications are that the infections in the controls were clearing up or had cleared up naturally, whereas those in the treated groups had only been suppressed and might or might not have developed to disease-producing proportions had the turkeys been retained alive for a longer period after medication had ceased. The whole question of possible relapse in its relation to the commencement of medication and time of infection requires further investigation on the lines followed by Joyner and Kendall (1955) in their study of the use of 2-amino-5-nitrothiazole in the control of histomoniasis. We are hoping to investigate this relationship during the current year. In view of possible relapse, it appears that continuous preventive medication is more effective in controlling the incidence of histomoniasis than is any practicable form of treatment against established infections. The possible side-effects produced in fowls by furazolidone are being studied at this laboratory. So far no indication has been found of any lowering of body weight in fowls that were fed furazolidone at concentrations in the feed of 0·005 or 0·02 per cent w/w over a twelve week period but marked testicular changes were noted (Cooper, private communication). Treatment should be restricted to non-breeding stock until such time as more is known of these effects.

CONCLUSIONS

Furazolidone at concentrations of 0·01 to 0·02 per cent w/w in the food is almost wholly effective in preventing histomoniasis induced by feeding *Heterakis* ova to turkeys when continuous medication is commenced before the ova are administered.

It is suggested that 0·015 per cent w/w furazolidone in the feed is a suitable concentration to use for preventive medication.

Treatment with 0·04 per cent w/w furazolidone in the food, delayed for ten days following the administration of *Heterakis* ova, prevented deaths from histomoniasis when continued for fourteen days.

It is suggested that treatment of established histomoniasis with a 0·04 per cent furazolidone medicated food be followed by a course of preventive therapy with 0·01 per cent in the food to combat disease arising from reinfection or relapse.

The action of furazolidone on histomonads is inhibitory, directly or indirectly, and the protozoa proceed to multiply if medication is withdrawn or is used at very low concentrations in the food.

Furazolidone at a concentration of 0·015 per cent in the food failed to prevent the development of disease caused by one species of turkey coccidium (suspected *E. adenoeides*).

Owing to the testicular changes observed in treated fowls, the medication of breeding stock is contra-indicated.

Further work is necessary to determine the relations existing between the recurrence of disease and the time and level of medication.

ACKNOWLEDGMENTS

Grateful acknowledgements are made to Dr. R. F. Gordon for his encouragement and to Dr. J. N. Oldham who read the manuscript and made many valuable suggestions for its improvement. We would also like to express our thanks to Miss M. Ward and Miss A. Brown for technical assistance and to Messrs. A. J. White, Ltd., and Messrs. May and Baker Ltd., for the supplies of furazolidone and 2-amino-5-nitrothiazole used in the experiments.

REFERENCES

Baker, A. D. (1933). *Sci. Agric.*, **13**, 356.
Cooper, D. M. (1955). *Private communication.*
Harwood, P. D., and Stunz, D. I., (1954). *J. Parasitol.*, **40**, Sect. 2 (Supplement), 24.
Horton-Smith, C., and Long P. L., (1951). *Vet. Rec.*, **63**, 507.
Joyner, L. P., and Kendall S. B., (1955). *Ibid.*, **67**, 180.
McGregor, J. K. (1953). *J. Amer. vet. med. Ass.*, **122**, 312.
Wehr, E. E. (1954). *J. Parasitol.*, **40**, Sect. 2 (Supplement) 26.

[*Received for publication, May 10th,* 1955]

SOME PATHOLOGICAL CHANGES IN THE CAT BRAIN

By

E. R. A. Cooper

Department of Anatomy, University of Manchester

and

I. Howarth

Department of Pharmacology, University of Cambridge

INTRODUCTION

During an investigation of the route of absorption of the cerebrospinal fluid*, sixty cat brains were examined histologically. Of these, four showed pathological change. The brains were fixed by carotid perfusion of formalin and immersion. The meninges were undisturbed in all the specimens, and none of the lesions was evident before sectioning, which was serial. No nervous symptoms were apparent during the short time (12 to 36 hours) the cats were under the authors' observation prior to experimentation.

RESULTS

Cat, K.22 (1.6 Kg.)

In this case a neoplasm (12 x 7 mm.)† occurred in the dorsolateral aspect of the left frontal lobe. The brain at this point was flattened at the surface, the minor fissures were disfigured, and the growth had infiltrated into the depths of the hemisphere (Fig. 1).

The characteristic lamination of the grey matter was not recognisable, the pyramidal neurones being displaced, degenerated or destroyed (Figs. 3A, 3B). Patches of fibrillary astrocytes projected strands into the cortex, dispersing the neurones and sometimes encircling small groups of these cells. The neoplasm encroached on the white matter and was in no way encapsulated (Fig. 1). Posteriorly there was patchy cystic degeneration, and a "roof" of glial fibres covered the surface of the hemisphere, obscuring the pia (Fig. 3A). The vascularity was not outstanding, but perivascular concentrations of astrocytes were observed in relation to some of the larger vessels.

The predominant cell in this growth was the fibrillary astrocyte (Fig. 2) and the appearances suggest that the neoplasm was a diffuse fibrillary astrocytoma (Elvidge, Penfield and Cone, 1937; Biggart, 1949).

Cat K.33 (1·9 Kg.)

An oval tumour (5 x 2·5 mm.)† occupied the grey cortex in the anterior region of the right frontal lobe of this cat. In the stained sections it appeared to the naked eye as a sharply defined pale area. Microscopically the peripheral cortical layers were evident and

* In the press.

† Measured from serial sections; actual size larger.

demarcated by a band of fibrillary glia from the underlying growth where only an occasional pyramidal neurone was recognisable. The deep aspect of the growth was also strictly limited by the band of fibrillary glia. Areas of calcification were scattered throughout the tumour and a few haemorrhagic patches were observed (Fig. 4).

The outstanding neoplastic cell was the round oligocyte (oligodendroglial cell) with a small, dense, ex-centric nucleus. These cells did not form a solid block, but were entangled in a mesh of fibrillary astrocytes (Fig. 5) which gave the tumour the appearance of the oligo-astrocytoma described by Bailey and Bucy (1929), Kwan and Alpers (1931) and Cooper (1935). Willis (1948) reports more growths of mixed or transitional structure than pure oligodendrogliomas in man.

The possibility that the pathological change in these two cases was a gliosis, following an inflammatory or degenerative lesion, has not been overlooked, but no vascular changes, no perivascular infiltrations and no signs of inflammation in the meninges were observed, and the rest of the cerebral tissue in both cases appeared normal (Figs. 3A, 3B). Biggart (1949) doubts whether a primary gliosis can occur.

Cat K.41 (2·3 Kg.)

A microscopic round tumour, about 0·2 mm. in diameter, was observed in this animal attached to the arachnoid membrane covering the frontal lobe, ventral to the right parasagittal sulcus. It was sessile and protruded towards the dura which was not involved. The tumour consisted of cells continuous with those of the arachnoid membrane, and they formed characteristic whorls, one of which contained a capillary and another a calcareous concretion. It was regarded as a leptomeningioma (Figs. 6 and 7).

Willis (1948) found symptomless meningiomas in routine human autopsies. Biggart (1949) states that histologically there is much resemblance between the architecture of a meningioma and a normal arachnoid granulation in man. He figures one of the latter which is similar to the authors' tumour. After a study of arachnoid granulations and villi in the cat, the authors regard their structure as an early stage of a meningioma perhaps arising from an arachnoid cell nest (Weed, 1914).

Cat K.54 (2·7 Kg.)

A discrete granuloma (0·6 x 0·3 mm.)† was present in the left temporal lobe just above the roof of the descending horn of the lateral ventricle (Fig. 8). It consisted of a central area of mononuclear epithelioid cells and a few capillaries. Around this were collections of small round cells, and a giant cell was observed here and there. The whole was encircled by glial fibres amongst which were small vessels filled with lymphocytes (Fig. 9). There was no meningitis. Ziehl-

† Measured from serial sections: actual size larger.

Neelsen staining did not reveal any acid-fast mycobacteria though this does not exclude the possibility of the structure being a tuberculoma.

The histological appearance resembles that of a pseudo-tuberculoma, described and figured by Cameron (1932, 1951), which develops around the egg of the lungworm (*Ælurostrongylus abstrusus*) in cats. Cameron states that in cats the eggs occasionally reach other parts of the body and die (1951). No egg was recognisable in this specimen, and since the nature of the research did not call for microscopic examination of the lungs, it is not known whether lungworm was present or not.

From this report it would seem that in the cat brain pathological changes may sometimes be similar to those occurring in the human brain. Willis (1948) states that gliomas in animals are "not as rare as the paucity of reports would suggest." Jungherr and Wolf (1939) describe two cases of brain tumour in cats in their survey, one an astrocytoma and the other a retinoblastoma. The meningioma and the granuloma are similar histologically to those found in man, but the cause of the latter could not be ascertained.

CONCLUSIONS

Four of a series of sixty cat brains examined in the course of a routine histological study showed pathological changes. A diffuse fibrillary astrocytoma and an oligodendroglioma were found in the frontal lobe of two animals; the other two lesions were an early leptomeningioma and a discrete microscopic granuloma.

ACKNOWLEDGMENTS

The authors wish to thank Professor G. A. G. Mitchell and Professor E. B. Verney for facilities enjoyed in their Departments in Manchester and Cambridge respectively; and the University of Manchester and the Medical Research Council for research grants received.

REFERENCES

Bailey, P., and Bucy, P. C. (1929). *J. Path. Bact.*, **32**, 735.
Biggart, J. H. (1949). *Pathology of the Nervous System.* Livingstone; Edinburgh.
Cameron, T. W. M. (1932). *J. Helminth*, **10**, 231; (1951). *The Parasites of Domestic Animals.* Black; London.
Cooper, E. R. A. (1935). *J. Path. Bact.*, **41**, 259.
Elvidge, A., Penfield, W., and Cone, W. (1937). Chapter VI in Vol. XVI of *Association for Research into Nervous and Mental Diseases*, Williams and Wilkins; Baltimore.
Jungherr, E., and Wolf, A. (1939). *Amer. J. Cancer*, **37**, 493.
Kwan, S. T., and Alpers, B. J. (1931). *Arch. Neurol. Psychiat.*, **26**, 279.
Weed, L. H. (1914-15). *J. med. Res.*, **26**, 21.
Willis, R. A. (1948). *Pathology of Tumours.* Butterworth; London.

[*Received for publication, June 14th,* 1955]

LEGENDS TO ILLUSTRATIONS

Fig. 1. K.22. Coronal section through left frontal lobe. X 12. H.E. a—arachnoid: b—dura: c—falx: d—medial fissure: e—white matter which is indistinct at the tumour site * to *.

Fig. 2. K.22. Tumour X 525. Mallory's phosphotungstic acid stain. a—astrocytes: b—astrocytic fibrillae: c—neurone.

Fig. 3. K.22. A. Tumour in left frontal lobe; lamination not recognisable. X 30. H.E.
B. Normal grey cortex from right frontal lobe showing lamination. X 30. H.E.
a—arachnoid: b—subarachnoid space: c—neuroglial fibres on surface of cortex: d—degenerative area: e—pia.

Fig. 4. K.33. Tumour X 45. H.E.
a—pia: b—superficial cortical layers: c—tumour: d—glial encapsulating band: e—calcified patches.

Fig. 5. K.33. Tumour X 675. Mallory's phosphotungstic acid stain. a—oligocytes: b—astrocyte: c—astrocytic fibrillae: d—calcified patch.

Fig. 6. K.41. Tumour in situ. X 125. H.E. a—tumour: b—arachnoid: c—pia.

Fig. 7. K.41. Tumour showing cell whorls. X 525. H.E. a—arachnoid: b—calcareous nodule: c—capillary.

Fig. 8. Granuloma in the temporal lobe above the descending horn of the lateral ventricle (a). X125. H.E.

Fig. 9. Granuloma X 300. H.E.
a—small round cells: b—giant cell: c—epithelioid cells: d—vessel containing lymphocytes: e—encircling glia: f—cerebral tissue.

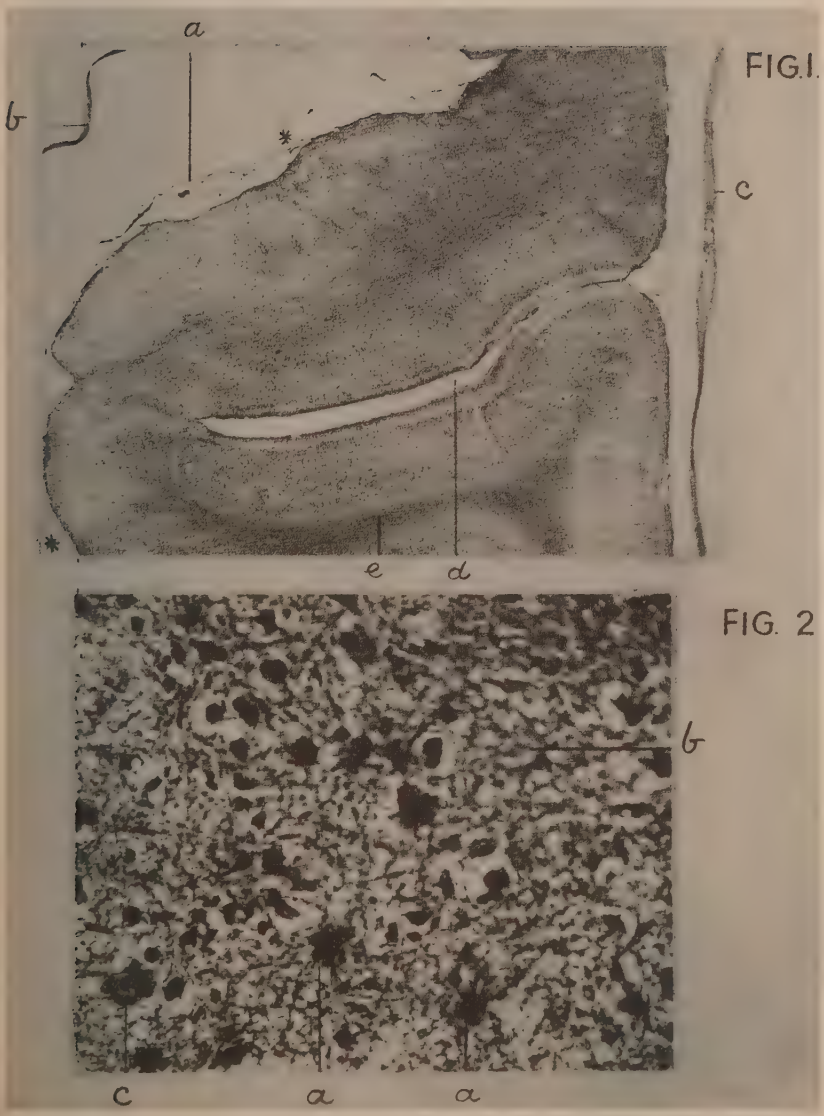


FIG. 1.

FIG. 2

To face page 38

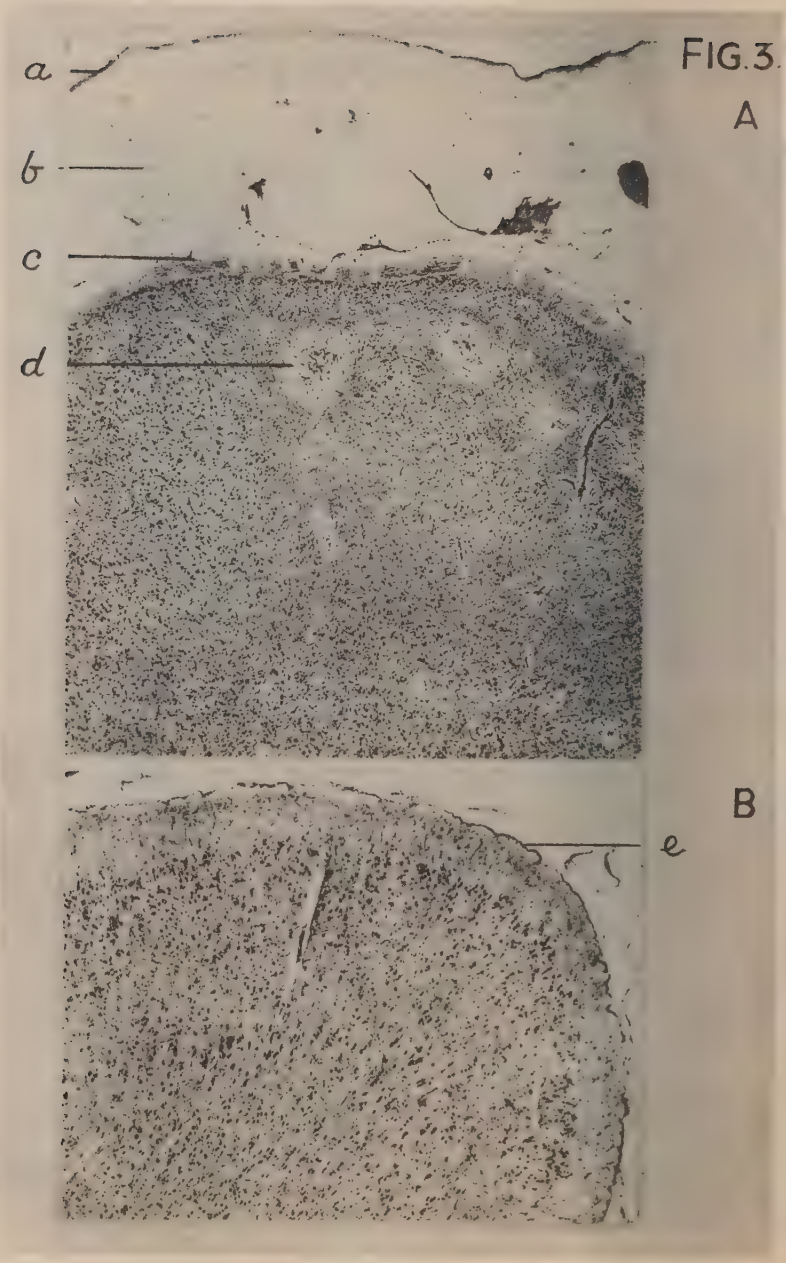


FIG. 3.

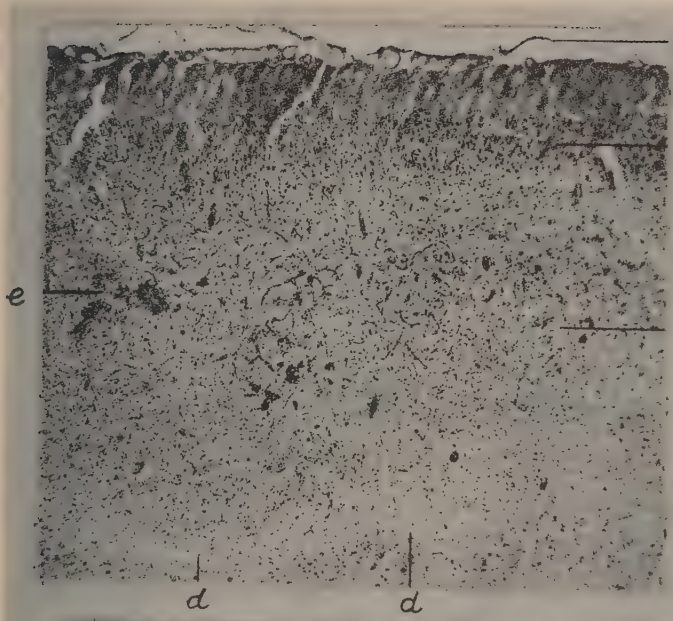


FIG. 4

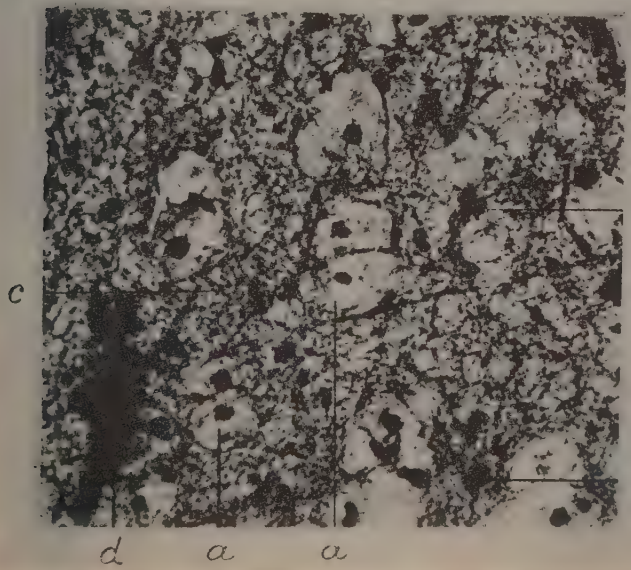


FIG. 5.

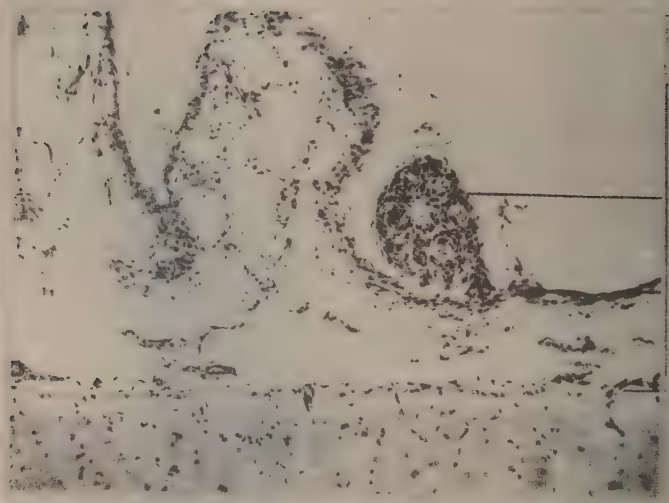


FIG.6.

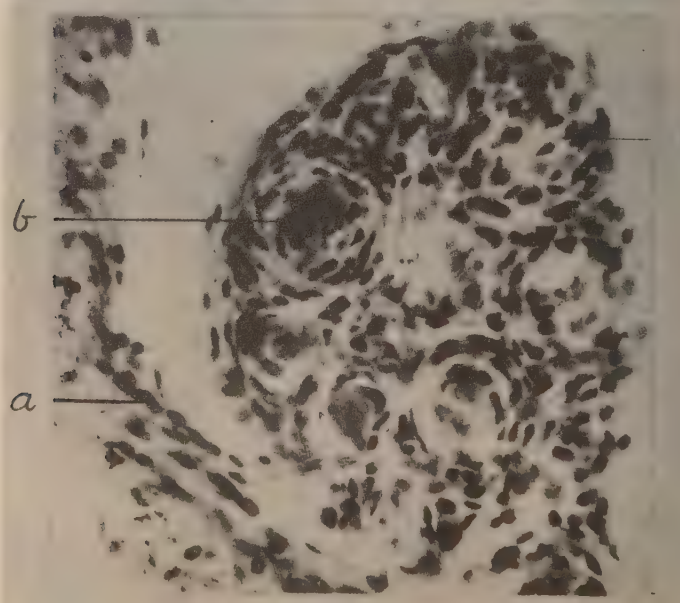


FIG.7

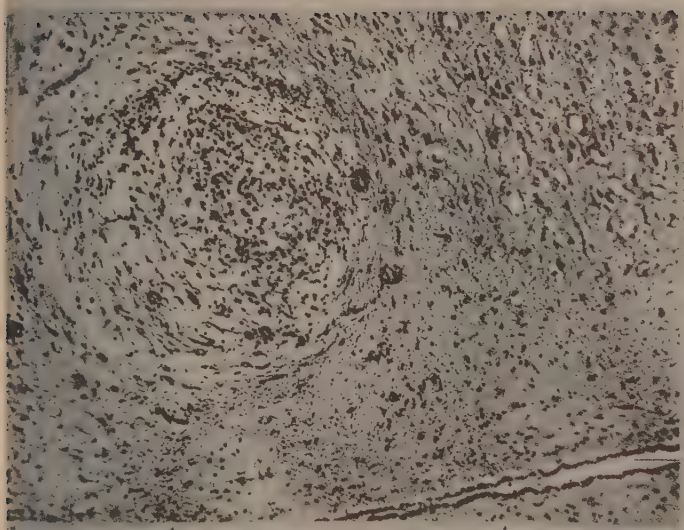


FIG.8

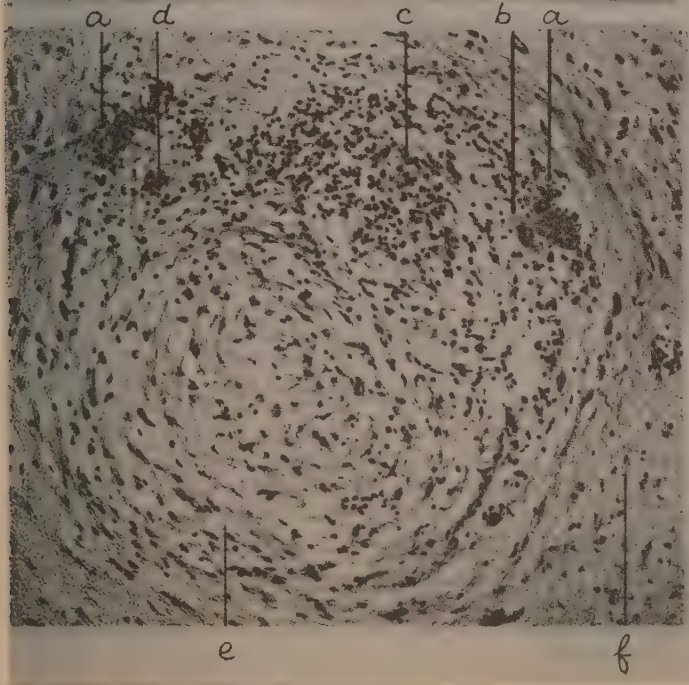


FIG.9

SULPHAQUINOXALINE POISONING IN CHICKENS

By

L. P. Joyner and S. F. M. Davies

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

It has been recognised that a condition characterised by haemorrhages in the body tissues and necrotic lesions in the spleen can occur in chickens treated for coccidiosis with sulphonamides, but the mechanism by which this syndrome is produced is still not understood (Davies, 1954). The administration of sulphonamides to laboratory animals has been found to lead to nutritional deficiencies which are attributed to disturbance of the intestinal bacterial flora (Daft and Sebrell, 1945). The deficiencies which have been recognised include a lack of biotin, pantothenic acid, vitamin E, vitamin K, folic acid (*L.casei* factor) and inositol.

A consideration of the nature of the lesions in chicks receiving sulphonamides suggests a similarity with the multiple haemorrhages associated with increased blood clotting time described by Dam (1935) in his original studies on vitamin K deficiency in chicks. This is supported by the work of Asplin and Boyland (1947) who demonstrated a hypoprothrombinaemia which could be corrected by the administration of synthetic vitamin K in chicks receiving Sulphamezathine.

From a study of the reports of field cases it appeared that the condition occurred rarely in young chicks and the most frequent reports of the haemorrhagic syndrome concerned birds older than four weeks (Davies, 1954). This is in agreement with the theory that the condition is due to a nutritional deficiency because absorption of the yolk sac taking place during the first weeks of life might render the bird less prone to such a deficiency. An alternative explanation is that the absorption and excretion of the drug are different in young and in older birds. Variations in absorption and excretion may also account for differences in individual susceptibility since only a proportion of the birds receiving the sulphonamides develop lesions (Davies and Kendall, 1953).

The investigation reported in this paper includes experiments on three factors, age of the bird, nutritional deficiency, differences in absorption and excretion of the drug, which may be implicated.

METHODS

The chicks used in these experiments were a strain of R.I.R. They were kept throughout the experiments in wire-floored heated hovers and were given a standard chick mash. The experiments were carried out during the spring, summer and autumn and no artificial light was supplied.

The sodium sulphaquinoxaline was a commercial preparation and was administered in the drinking water in a concentration of 0·0645 per cent, equivalent to 0·06 per cent sulphaquinoxaline. The dose usually recommended for practical use has been reduced in Britain from 0·06 per cent sulphaquinoxaline to 0·04 per cent, but in these experiments the higher concentration was given for seven days in order to reproduce the condition with greater regularity.

The synthetic vitamin K analogue (2 methyl-/: 4-naphthohydroquinone) was dissolved in arachis oil and injected subcutaneously. The vitamin K_1 was diluted with sterile distilled water before injection.

Blood clotting times were estimated by the method of Cruse and Moses (1945) and the concentrations of sulphaquinoxaline in blood were estimated by the method of Bratton and Marshall (1939) after precipitation of the protein with trichloracetic acid. The colour densities were estimated with a photoelectric absorptiometer.

RESULTS

The Susceptibility of Birds of Different Ages.

A group of day-old chicks was set aside. At intervals, groups of ten birds were withdrawn and given 0·0645 per cent sodium sulphaquinoxaline in the drinking water. After seven days the blood clotting time of the birds was determined and compared with that of a group of 5 normal birds of the same age. The drug was removed on the seventh day and on the ninth day the birds were killed. The susceptibility to sulphaquinoxaline poisoning of samples of chicks from the same stock at different ages was thus tested. The number of deaths during treatment was recorded and at the post-mortem examinations the occurrence of fluid in the body cavity, muscle haemorrhages and spleen lesions were noted, these being, in our experience, the most characteristic manifestations of toxicity. The results which are recorded in Table 1 show that in birds of all ages the administration of the drug caused an increase in the blood-clotting time, but the incidence of lesions and mortality was highest in birds aged from 4 to 8 weeks.

A possible explanation of the above result is that the absorption and excretion of the drug in the younger birds followed a different pattern from that in the older birds. The experiment was therefore repeated but in addition the concentration of the drug in the blood of the birds was estimated on the seventh day of treatment. Groups of ten birds were withdrawn from a common stock at intervals and given 0·0645 per cent sodium sulphaquinoxaline for seven days. The birds were killed after a further 2 days without drug and examined post-mortem.

TABLE 1

THE SEVERITY OF SULPHAQUINOXALINE TOXICITY IN CHICKS OF DIFFERENT AGES

Age in days	Number of birds showing:			Number of deaths	Total affected out of each group of 10	Blood clotting time of treated birds in minutes		Blood clotting time of similar normal birds in minutes	
	Fluid in the body cavity	Muscle haemorrhage	Spleen lesions			Mean	Range	Mean	Range
7	0	0	0	0	0	7·8	3·3–11·7	1·9	1·0– 2·8
12	0	0	1	1	1	11·3	3·0–20·8	1·8	0·8– 2·5
14	1	0	3	0	3	9·9	3·5–20·8	2·2	1·1– 2·7
18	2	0	2	1	3	15·3	5·2–36·7	2·7	1·3– 5·3
21	0	0	1	0	1	9·9	2·8–20·4	2·9	1·4– 5·0
25	1	0	1	2	2	17·4	3·5–30·9	2·6	1·6– 3·3
28	2	1	4	5	5	23·0	8·3–30·0	3·4	2·6– 3·4
32	1	1	7	3	7	21·7	13·5–30·0	6·7	3·5–13·0
35	4	0	6	1	6	15·0	5·45–30·0	5·1	3·5–10·9
39	0	0	3	0	3	10·4	3·4–25·4	6·15	4·4–10·0
46	2	0	4	0	5	7·5	2·1–17·4	3·5	1·4– 4·4
49	3	0	6	0	6	14·1	5·9–30·0	3·7	1·8– 5·8
53	4	2	6	0	7	8·7	5·4–19·9	3·8	1·3– 5·9
56	0	0	8	0	8	8·1	2·7–17·8	5·1	1·8– 5·7
61	1	4	5	0	5	5·0	1·4– 7·9	2·1	1·0– 2·7
63	0	0	3	0	3	7·4	2·0–18·9	4·9	2·8–10·0
67	2	0	6	0	7	6·0	3·7–14·7	4·6	1·7– 9·4
70	1	0	7	0	7	5·6	1·1– 9·3		
74	1	0	4	0	5	4·4	2·0–14·2		

The total number of birds with typical toxic changes have been recorded in Table 2. The incidence of accumulations of fluid in the body cavity and of spleen lesions has also been noted because these changes were the commonest. The results recorded in Table 2 show

TABLE 2

SULPHAQUINOXALINE BLOOD LEVELS AND TOXICITY IN CHICKS OF DIFFERENT AGES

Age of chicks at commencement of treatment	*Total sulphaquinoxaline concentration in blood mg. per 100 ml.*		*Number of birds showing*		*Total affected out of groups of ten chicks*
	Mean	*Standard dev.*	*Fluid in body cavity*	*Spleen lesions*	
14 days	8·45	1·6	2	2	3
21 days	9·9	1·3	0	5	5
28 days	9·1	1·3	4	6	8
42 days	7·9	0·28	0	4	6
63 days	8·3	1·2	0	2	2

that there was no significant difference between the drug levels in the different groups although the evidence of toxicity was most marked in the chicks aged three to six weeks.

In subsequent experiments it was decided to use five-to-six weeks old chicks, at which age they consistently showed high susceptibility.

The Relationship between the Concentration of Sulphaquinoxaline in the Blood and Clotting Time

Most of the nutritional deficiencies which occur in animals receiving sulphonamides are attributed to an inhibition of the intestinal bacterial flora (Daft and Sebrell, 1945). It would be expected that a period of time must elapse between the commencement of drug administration and the appearance of the symptoms of deficiency to allow for exhaustion of nutritional reserves. It was therefore of interest to observe the rate at which the blood clotting time increased and at the same time to record the concentration of sulphaquinoxaline in the blood.

In the experiment recorded in Fig. 1 the blood-clotting times of twelve 6-weeks old chicks were recorded at intervals for seven days. During this time they were given 0·0645 per cent sodium sulphaquinoxaline in the drinking water for 72 hours. The concentration of the total amount of drug in the blood was also determined. It will be seen that there was a very close correlation between the concentration of drug in the blood and the clotting time, the latter increasing almost as soon as the drug began to appear in the blood. Withdrawal

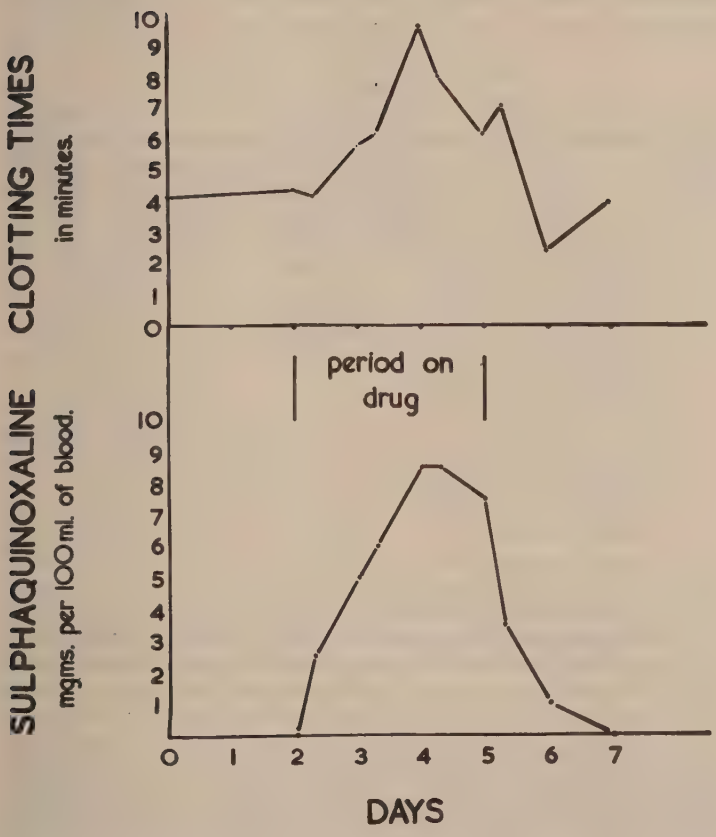


Fig 1. The relationship between the concentration of sulphaquinoxaline in the blood and blood-clotting time.

of the drug was promptly followed by a reduction in the clotting time. This phenomenon is suggestive of a direct toxic effect rather than indirect inhibition of the intestinal flora.

The Effect of Dietary Supplements on the Toxicity of Sulphaquinoxaline

The effect of synthetic vitamin K on sulphamezathine toxicity was investigated by Asplin and Boyland (1947) who found that intestinal haemorrhages caused by the drug were not completely

reduced by the vitamin, although the blood clotting time was restored to normal. It seemed important to study the effect of this vitamin on chicks receiving sulphaquinoxaline. In the experiments recorded in Table 3, three groups of twelve chicks were given 0·0645

TABLE 3

THE EFFECT OF VITAMIN K ON THE TOXIC CHANGES DUE TO SULPHAQUINOXALINE

Number of chicks	*Treatment*	*Clotting time (Average in minutes)*		*Number of Chicks with spleen lesions on 9th day*	*Total number chicks affected*
		Day 0+4	*Day 0+7*		
12	0·0645 per cent sod. sulphaquinoxaline in drinking water. Days 0 to 7 incl.	9·85	7·83	5	8
12	0·0645 per cent sod.sulphaquinoxaline in drinking water for 7 days. Vit. K. analogue 10 mg. sub-cut. Days 0 to 0+6 incl.	4·23	1·78	4	5
12	0.0645 per cent sod. sulphaquinoxaline in drinking water for 7 days. Vit. K_1 4 mg./Kg. intravenous Days 0 to 0+6 incl.	1·73	2·28	2	6

per cent sodium sulphaquinoxaline in the drinking water for seven days. One group was also given six daily intravenous injections of 4 mg. vitamin K_1, the second group daily subcutaneous injections of 10 mg. of synthetic vitamin K analogue and the final group sulphonamide only.

On the ninth day after the commencement of treatment all the birds were killed and examined. Spleen lesions were the commonest: a few birds showed accumulations of fluid in the body cavities and haemorrhages in other organs. For simplicity of recording, the total numbers of birds affected and the numbers with spleen lesions have been noted in Table 3.

The results show that although the blood clotting time was reduced, neither vitamin preparation had any effect upon the incidence of lesions.

A further experiment was set up to compare the blood clotting time of normal chicks, chicks receiving vitamin K analogue only, and chicks receiving sulphaquinoxaline plus the vitamin. A similar group of birds was given sulphaquinoxaline only. The results recorded in Table 4 show that the blood clotting time of the normal birds was not affected by the vitamin and that of the birds receiving sulphaquinoxaline plus the vitamin K was reduced to normal levels. The vitamin thus

has a direct neutralizing effect upon the increased clotting time caused by the drug.

TABLE 4

THE EFFECT OF VITAMIN K ON THE BLOOD-CLOTTING TIME OF NORMAL AND SULPHAQUINOXALINE-TREATED CHICKS

Number of birds	*Treatment*	*Blood-clotting time average in minutes*			*Number of chicks with spleen lesions*	*Total number of chicks affected*
		Day 0+3	*Day 0+6*	*Day 0+8*		
12	Normal controls	3·7	3·0	2·7	Nil	Nil
12	20 mg. Vit. K (synthetic) S/cut. Day 0, 0+3 and 0+6	2·6	3·0	1·6	Nil	Nil
12	0·0645 per cent sod. sulphaquinoxaline Day 0 to 0+7 incl. 20 mg. Vit. K (synthetic) S/cut day 0, 0+3 and 0+6	3·9	2·3	1·8	1	4
12	0·0645 per cent soda sulphaquinoxaline Day 0 to 0+7 incl.	10·5	10·1	4·7	2	6

It is possible that the toxic effects of sulphaquinoxaline are due to the antagonism of some essential metabolite, or to a deficiency of a product of the metabolism of the intestinal bacterial flora other than vitamin K. In the experiments recorded in Table 5 one group of chicks receiving 0·0645 per cent sodium sulphaquinoxaline in the drinking water was given para-amino benzoic acid which has been found by Black, Overman, Elveljem and Link (1942) and Kornberg. Daft and Sebrell (1944b) to reduce the hypoprothrombinaemia, caused by the administration of sulphonamides in the diet of laboratory rodents. Other groups of chicks were given a series of vitamins, or liver extract in combination with vitamin K. None of the substances tested was shown to be capable of preventing the lesions.

DISCUSSION

The occurrence of subcutaneous and muscular haemorrhages with lesions in the spleen, intestines, heart and other organs, together with increased blood clotting times in fowls under normal management have been associated with a deficiency of vitamin K. Goldhaft and Wernicoff (1954) described such a condition which could not always be ascribed to the use of sulphaquinoxaline. They reported that mortality could be controlled in some cases by the administra-

TABLE 5

THE EFFECT OF DIETARY SUPPLEMENTS AND INJECTED VITAMINS ON SULPHAQUINOXALINE TOXICITY

Number of chicks	*Treatment*	*Number of chicks with spleen lesions only*	*Total number of chicks affected*
20	20 mg. Vit. K (synthetic) 5 mg. Riboflavin 5 mg. Vitamin B_6 2 mg. Vitamin B_1 5 mg. Panthothenic acid } days 0+2 and 0+4	3	6
20	20 mg. Vit. K (synthetic) Vit. A 1000 I.U. Vit. D_3 200 I.U. } day 0—1 and 0+2	8	12
20	20 mg. Vit. K (synthetic) 100 mg. Liver extract } days 0—1, 0+2 and 0+4	6	13
9	20 mg. Vit. K (synthetic) 10 mg. Ascorbic acid } days 0, 0+2, 0+4 and 0+6	1	3
22	p. amino benzoic acid 0·1 per cent in food	4	16

tion of Menadione (Vit. K analogue) in the drinking water. Griminger, Fisher, Morrison, Snyder and Scott (1953) and Hare, Anderson and Weakley (1953) reported similar conditions in birds fed for several weeks on vitamin K deficient diets. It seems likely that the condition in birds receiving simplified rations described by Anderson, Hare, Bletner, Weakley and Mason (1954) was also of nutritional origin because the multiple haemorrhages which occurred took several weeks to develop and the severity was not increased by the administration of low doses of sulphaquinoxaline; blood clotting times and mortality were reduced by the administration of Menadione. There is no doubt that absence of vitamin K from the diet causes marked pathological changes and the resulting increased blood clotting time can be reproduced with sufficient regularity to provide the basis for pharmacological assay of the vitamin (Almquist, 1941).

Vitamin K deficiency can also arise in animals given sulphonamides in the diet; the increased blood clotting time and tissue haemorrhages which are the main symptoms of the deficiency occur after a period of several weeks (Kornberg, Daft and Sebrell, 1944a). It would be expected therefore that such a condition can also arise in chicks treated with sulphonamide drugs for coccidiosis provided that the concentration of the drug is sufficiently high to influence the bacterial flora of the gut for a sufficient length of time.

The lesions due to sulphaquinoxaline poisoning described by Davies and Kendall (1953) and Davies (1954), however, develop

within 5 to 7 days of the commencement of therapy and the present observations have shown that the effect upon blood clotting becomes apparent as soon as the drug is absorbed.

Growth inhibition and hypoprothrombinaemia which occur in laboratory rodents given sulphonamides in the diet were shown by Black, *et. al.* (1942) and Kornberg *et. al.* (1944b) to be reduced by the administration of para-amino-benzoic acid which is well known as an antagonist to the bacteriocidal effects of sulphonamides. It will be seen from table 5 that no such effect could be demonstrated in chicks receiving sulphaquinoxaline. It seems likely, therefore, that the drug does not in this instance interfere with the absorption of vitamin K by modifying the bacterial flora of the gut. Nor is it likely that the drug acts as an antagonist to the vitamin, because the lesions were not reduced by the administration of either pure or synthetic vitamin K at the same time as the sulphaquinoxaline.

The only effect which vitamin K has on sulphaquinoxaline poisoning is to reduce the blood clotting time to normal; it has no effect upon other manifestations of toxicity viz: the tissue lesions. It is unlikely, therefore, that the toxicity is due solely to a shortage of vitamin K either because of inhibition of the intestinal bacterial flora or because of a deficiency of the vitamin in the diet.

In the field two haemorrhagic conditions may exist; one caused by vitamin K deficiency, and the other by sulphaquinoxaline toxicity. The relative importance of the two conditions can only be assessed through fuller investigations both in the laboratory and in field outbreaks.

CONCLUSIONS

It has been shown that the greater susceptibility of chicks at the age of 4 to 8 weeks to sulphaquinoxaline cannot be attributed to any greater absorption of the drug at that age. Furthermore, the increase in the blood clotting time which follows the administration of the drug does not vary with the age of the birds.

There was a close correlation between the concentration of sulphaquinoxaline in the blood and the blood clotting time; the latter increased almost as soon as the sulphaquinoxaline began to appear in the blood and diminished promptly after withdrawal of the drug.

The administration of vitamin K or synthetic analogues of vitamin K reduced the blood clotting time of birds receiving sulphaquinoxaline but did not reduce the incidence of lesions.

Dietary supplements of para-aminobenzoic acid or injections of ascorbic acid, vitamins A, B_1, B_6, D, K, riboflavine, and pantothenic acid did not reduce the incidence of lesions of sulphaquinoxaline poisoning.

ACKNOWLEDGMENT

We are indebted to Messrs. Geigy Ltd., for the vitamin K_1.

REFERENCES

Almquist, H. J. (1941). *J. Ass. off. agric. Chem. Wash.*, **24**, 405.
Anderson, G. C., Hare J. H., Bletner, J. K., Weakley, C. E. and Mason, J. A. (1954). *Poultry Sci.*, **33**, 120.
Asplin, F. D., and Boyland, E. (1947). *Brit J. Pharmacol.*, **2**, 79.
Black, S., Overman, R. S., Elvehjem, C. A. and Link, K. P., (1942). *J. biol. Chem.*, **145**, 137.
Bratton, A. C., and Marshall, E. K. Jr. (1939). *Ibid.*, **128**, 537.
Cruse, T. K., and Moses, C (1945). *J. lab. clin. Med.*, **30**, 631.
Daft, F. S., and Sebrell W. H., (1945). *Vitamins and Hormones*. **3**, 49. New York.
Dam, H. (1935). *Biochem. J.*, **29**, 1273.
Davies, S. F. M. (1954). *Tenth World's Poultry Congress, Edinburgh*. p. 275.
Davies, S. F. M., and Kendall, S. B. (1953). *Vet. Rec*, **65**, 85.
Goldhaft, T. M., and Wernicoff, N. (1954). *Tenth World's Poultry Congress, Edinburgh*, p. 278.
Griminger, P., Fisher, H., Morrison, W. D., Snyder, J. M., and Scott, H. M. (1953). *Science*, **118**, 379.
Hare, J. H., Anderson, C. E., and Weakley, Jr., C. E. (1953). *Poultry Sci.*, **32**, 904.
Kornberg, A., Daft, F. S. and Sebrell, W. H. (1944a). *Pub. Health Rep.* **59**, 832; (1944b). *J. biol. Chem.*, **155**, 193.

[*Received for publication, June 23rd,* 1955]

EXPERIMENTAL STREPTOCOCCAL MASTITIS

XI. Immunological Studies in the Cow

By

D. G. Howell, I. M. Smith, H. H. Holman and I. H. Pattison

Agricultural Research Council, Field Station, Compton, Berkshire.

INTRODUCTION

Earlier studies at this Field Station, using the goat as the experimental animal, showed that a parenterally administered vaccine gave some protection against experimentally produced mastitis. Thus, a vaccine of living *Streptococcus agalactiae*, injected by various routes, gave readily demonstrable protection against an intramammary test dose of the homologous strain (Holman, Pattison and Gordon, 1952), and a polyvalent formolised vaccine containing each of Lancefield's four serological types, injected intraperitoneally or subcutaneously, gave measurable protection against an intramammary test dose of any one of these strains (Pattison and Holman, 1953).

These findings with goats were sufficiently encouraging to warrant similar studies with cows, and this paper records the results of two experiments in which cows vaccinated subcutaneously with formolised broth culture of *Str. agalactiae* were exposed to an intramammary test dose of the homologous strain.

MATERIALS AND METHODS

Animals. The cows had a similar history to those used in previous experiments (Howell, Pattison, Holman and Smith, 1954[a]) and were housed in isolation at this Field Station. Because of the resistance to intramammary infection with *Str. agalactiae* of cows with a serum titre of staphylococcal hyaluronidase inhibitor (Howell, Pattison, Holman and Smith, 1954[b]), animals with this inhibitor were distributed as evenly as possible between vaccinated and control groups. Two experiments were carried out. In the first, 22 cows were vaccinated and 22 were used as controls; in the second, five of the vaccinated cows were rechallenged and five additional controls were introduced.

Strain of Str. agalactiae. The bovine strain A.100, Lancefield group-B type II, was used for vaccination and for challenge.

Vaccine. Vaccine was freshly prepared for each inoculation by adding formalin to an 18-hour Todd-Hewitt broth culture to give a final concentration of 0.35 per cent; a sample plated out 24 hours later showed that the vaccine was non-viable. Vaccinated cows were given 10 ml. of vaccine subcutaneously each week for four weeks and were then rested for three weeks; two more weekly doses followed, and the intramammary test dose was injected after a second rest period of three weeks. Control cows received a similar series of 10 ml. injections of formolised broth.

Intramammary challenge. The test doses used in the three experiments were prepared and injected as described by Howell, *et al.* (1954[a]). The

doses used were approximately 10^5, 10^7 and 10^8 viable organisms, the calculated counts being 5×10^5, 1.5×10^7, and 5×10^8. Howell, *et al.* (1954[a]) showed that the minimal infecting dose of strain A.100 for cows not showing a serum titre of staphylococcal hyaluronidase inhibitor was about 5×10^4 viable organisms. The above doses were therefore about 10, 300, and 10,000 minimal infecting doses.

Tests for mastitis. The tests used were the bacterial and neutrophil content of the milk, the appearance of the milk, and the Whiteside test as described by Howell, *et al.* (1954[a]).

Detection of antibody. A serum agglutination test (Holman, *et al.*, 1952), using strain A.100 as the antigen, and a test for streptococccal hyaluronidase inhibitor were carried out fortnightly during the immunisation process on the five animals which received a challenge of 10^6 organisms and on the 12 that received a challenge of 10^7. Prior to challenge the serum of all animals was tested for staphylococcal hyaluronidase inhibitor by the method described by Howell (1954).

Quarters inoculated with Str. agalactiae. The test dose was inoculated into one quarter only of each cow. The quarters inoculated were selected as being negative on two occasions to bacteriological, neutrophil, and Whiteside tests.

Measurements made after inoculation of the test dose. The tests for mastitis were carried out twice daily on milk from the inoculated quarter, and once weekly on all other quarters. Pre-infection measurements were made on all animals for at least one week, and each experiment continued for a minimum of three weeks after inoculation of the test dose. The streptococci recovered from cows after challenge were identified serologically—especially when their appearance in culture was erratic—by the method described by Pattison, Matthews and Howell (1955).

Statistical. Yates's modification was used in the estimation of χ^2. The results for the exact tests were read from Mainland's tables (Mainland, 1948).

RESULTS

The Effects of Vaccination.

Clinical. The first and second vaccinations with formolised whole broth culture—but not with formolised broth—caused slight dullness and some rise in temperature in one or two cows on the day of vaccination, with a small decrease in milk yield in these animals on the following day. However, comparison of total weekly milk yields of vaccinated and of control cows failed to show a significant difference attributable to vaccination. Swellings occurred occasionally at the site of vaccination, but had disappeared before inoculation of the test dose.

Development of antibody. The progressive but erratic development of agglutinating antibody to strain A 100 during the immunisation process, and the appearance of streptococcal hyaluronidase inhibitor titres, are shown in Table 1. The serum titres of staphylococcal hyaluronidase inhibitor were not influenced by vaccination, although in one or two cows in both the vaccinated and control groups they

did appear at minimal titres during the period of vaccination; their appearance, however, was not related to any clinical manifestation. The titres for these inhibitors recorded in Table 2 were those determined just before injection of the intramammary test dose.

TABLE 1

SERUM TITRES

THE FREQUENCY DISTRIBUTION TO STREPTOCOCCAL HYALURONIDASE INHIBITOR AND TO *STR. AGALACTIAE* A.100 AGGLUTININS IN 17 COWS VACCINATED WITH FORMOLISED A.100 VACCINE (SEE TEXT) AND EXPOSED TO AN INTRAMAMMARY TEST DOSE OF EITHER 10^6 OR 10^7 LIVING ORGANISMS OF STRAIN A.100.

Week of experiment	*Vaccinations*	*Titre of Strept. hyaluronidase inhibitor*					*Titre of A.100 agglutinins*						
		<1:16	1:16	1:32	1:64	1:128	<1:4	1:8	1:16	1:32	1:64	1:128	1:256
0 1	1st 2nd	17	0	0	0	0	17	0	0	0	0	0	0
2 3	3rd 4th	17	0	0	0	0	10	6	0	1	0	0	0
4 5	— —	13	1	2	1	0	2	2	5	4	2	2	0
6 7	5th 6th	14	0	1	1	1	4	0	5	5	1	1	1
8 9	— —	4	1	6	4	2	0	0	2	6	4	3	2
10 11	*test dose* —	7	0	8	1	1	1	2	0	5	7	2	0
12	—	11	0	3	3	0	2	1	3	5	6	0	0

Results following Injection of the Intramammary Test Dose

Presentation of Data. A study by one of us (I.H.P.) of the histopathology of experimental streptococcal mastitis in the cow indicated that the disease was identical in nature, and in speed of develop-

ment, with that previously studied in the goat (Pattison, 1951). In immunological experiments with goats (Holman, *et al.* 1952) reasons were given for selecting the second week after injection of the test dose as the most suitable period for examining in detail, and for comparing, the reactions of vaccinated and of control animals. The period of 14 days after infection with *Str. agalactiae* covers what have been called "transient" infections in the cow (Howell, *et al.*, 1954[a]). For these reasons we have based our conclusions in this paper on a comparison of the reactions of vaccinated and control cows during the first two weeks after intramammary infection, and the results—in the form of daily average measurements for each animal for the first and second weeks after infection —are given in Table 2.

Differences between vaccinated and control groups. A direct comparison between vaccinated and control cows is given by the grand totals in Table 2. There is an obvious difference in bacterial and neutrophil counts between the groups. The count of *Str. agalactiae* in the milk of the vaccinated cows for the two weeks averaged 9.0 thousand and 4.0 thousand per ml., compared with 23.4 thousand and 13.9 thousand for the controls. Further, at the end of the second week *Str. agalactiae* was absent from the milk of 13 of 22 vaccinated cows, but from only two of an equal number of control cows. These figures give χ^2 as 10.1 and p as less than 0.001.

Neutrophil counts in the vaccinated group averaged 4.6 millions and 1.0 million for the two weeks, compared with 5.6 millions and 2.5 millions in the control group. If a comparison is made between the cows in each group with a count of 500,000 neutrophils or less per ml. of milk in the second week, then 15 of 22 vaccinated cows are included as against three of 22 control cows. These results give a value for χ^2 of 11.4 and p is less than 0.001.

The other totals given in Table 2—for the appearance of milk, the Whiteside test and the milk yield from the infected quarter—show no obvious advantage in favour of the vaccinated animals. The larger percentage milk yield for vaccinated cows in the second week is not significantly different from the percentage yield of the controls, and treatment of the actual yields of the infected quarters for each cow by an analysis of covariance failed to show a significant difference between vaccinated and control animals. However, one significant difference between yields of infected quarters of vaccinated and control cows is apparent in the table, namely the frequency with which the yield in the second week after the test dose represented a gain or loss compared with that in the first week. Thus, 17 of 22 vaccinated cows showed a gain in the second week, compared with only five of 22 controls, giving χ^2 value of 13 and p less than 0.001. Additional figures showed that in the third week, in 11 vaccinated cows the gain increased, and in 11 control cows

the fall was progressive, indicating that there was less functional damage and more rapid recovery in the infected quarters of the vaccinated group.

Differences due to the size of the intramammary test dose. Howell *et al.* (1954[a]) found that an intramammary dose of 10^3 living *Str. agalactiae* strain A 100 failed to produce mastitis in any cows, and that the establishment of infection with doses of 10^4, 10^5, and 10^6 was partly dependent on the presence of an inhibitor to staphylococcal hyaluronidase detectable in the cow's blood (Howell, *et al.*, 1954[b]).

The figures in Table 2 show that *Str. agalactiae* was recovered for at least one week from the milk of all the 17 vaccinated cows given a dose of more than 10^5 living organisms. By contrast, *Str. agalactiae* was not recovered at all from four of the five vaccinated cows challenged with 10^5 living organisms. These two results are not homogeneous ($p = .0084$).

This marked difference between groups of vaccinated cows exposed to test doses of different size, raised the question of the permissibility of measuring the effect of vaccination by using the total figures for all cows irrespective of the size of test dose. The results given in Table 2 were therefore re-examined, and a comparison was made between the vaccinated and control animals exposed to the two larger test doses. It was found that the counts of *Str. agalactiae* and of neutrophils per ml. of milk at the end of the second week, and the increase in milk yield in the second week, still showed a highly significant difference between vaccinated and control cows. It was concluded, therefore, that even when the good results following the smallest test dose were disregarded there was still a clearly significant bias in favour of the vaccinated cows.

The size of the test dose influenced the measurements recorded in Table 2 in two other ways. Firstly, the milk yield of both vaccinated and control animals decreased as the number of *Str. agalactiae* in the test dose increased. Secondly, in the control groups the count of *Str. agalactiae* per ml. of milk in the second week in staphylococcal hyaluronidase inhibitor negative cows averaged only 5.7 thousand in the 10^5 or 10^7 dose animals compared with 53.3 thousand in the animals given 10^8 living organisms.

Differences due to detectable antibodies. It was reported by Howell, *et al.* (1954[b]) that cows with a serum titre of an inhibitor to staphylococcal hyaluronidase showed some degree of resistance to an intramammary test dose of *Str. agalactiae.* From Table 2 it will be seen that all of 11 staphylococcal hyaluronidase inhibitor-negative control cows became infected, and that in the second week five of them had A 100 counts exceeding 21,000 organisms per ml. of milk. By contrast, none of 11 inhibitor-positive control cows has A 100 counts above 11,400 organisms per ml. of milk in the second

TABLE

RESULTS OF MILK EXAMINATION ON PROTECTED AND UNPROTECTED COWS. MARY TEST DOSE OF A.100

Vaccinated

Test dose of A100	*Cow No.*	*Reciprocals of final preinfection blood titres*			*Results for milk from infected quarter*									
					% Milk yield		*Neutrophil count 10^6/ml.*		*A100 count 10^3/ml.*		*Whiteside test units*		*Milk appearance units*	
		AH Staph.*	*AH* Str.*	*A100 Agg.*	*Wk.** 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*
10^5	1	0	32	64	94	95	1·5	0	0	0	2	0	0	0
	2	0	32	64	89	98	0·1	0	0	0	2	1	1	0
	3	0	64	64	80	83	0·1	0	0	0	2	2	1	0
	4	0	64	64	96	105	1·6	0	0	0	2	1	1	0
	5	0	128	64	57	40	3·8	0·1	7·2	0	4	2	4	1
			Total		416	421	7·1	0·1	7·2	0	12	6	7	1
			Average		83	84	1·4	0	1·4	0	2	1	1	0
10^7	11	0	32	8	24	26	6·5	3·8	32·4	0·9	3	3	5	4
	12	0	32	64	61	70	7·5	0·2	3·1	0	3	1	4	1
	13	0	64	8	52	39	2·1	0·3	48·5	14·2	4	3	4	3
	14	32	0	32	87	86	4·8	0·1	2·7	0	2	1	2	0
	15	32	32	4	73	78	6·0	2·1	3·0	7·8	3	2	1	1
	16	64	0	32	58	76	6·5	0·9	4·4	0·2	4	2	4	1
	17	64	0	128	78	87	6·1	0·3	0·5	0·0	3	2	3	1
	18	128	0	32	82	77	3·2	5·1	2·7	7·3	4	3	1	2
	19	128	0	32	61	75	6·1	1·2	2·0	5·0	3	2	3	1
	20	128	0	128	69	76	9·1	0·3	0·1	0·0	3	2	2	0
	21	128	32	32	67	72	5·6	4·9	4·5	6·3	3	4	3	3
	22	128	128	64	78	73	5·1	0·4	1·0	0·0	3	1	2	0
			Total		790	835	68·6	19·6	104·9	41·7	38	26	34	7
			Average		66	70	5·7	1·6	8·7	3·5	3	2	3	1
10^8	35	0	0	64	35	59	6·9	0·2	23·3	38·6	4	2	3	0
	36	0	32	32	64	68	2·6	0·2	1·5	0	4	2	2	1
	37	0	64	32	57	81	4·7	0·5	14·0	7·9	4	2	3	1
	38	0	128	32	25	31	5·4	0·9	30·9	0	4	2	4	1
	39	128	128	64	42	61	4·9	0·3	15·8	0	4	2	3	0
			Total		223	300	24·5	2·1	85·5	46·5	20	10	15	3
			Average		45	60	4·9	0·4	17·1	9·3	4	2	3	1
			Grand total		1429		100·2		197·6		70		56	
			Average		65		4·6		9·0		3·2		2·5	
			Grand total			1556		21·8		88·2		42		21
			Average			71		1·0		4·0		1·9		0·9

*Anti-Hyaluronidase

**Milk yield as % of preinfection yield.

2

DAILY AVERAGES FOR THE FIRST AND SECOND WEEKS FOLLOWING AN INTRAMAM-

Controls

Cow No.	Reciprocals of final preinfection blood titres			Results for milk from infected quarter									
				% Milk yield		Neutrophil count 10^6/ml.		A 100 count 10^3/ml.		Whiteside test units		Milk appearance units	
	AH* Staph.	AH* Str.	A 100 Agg.	Wk.** 1	Wk. 2	Wk. 1	Wk. 2	Wk. 1	Wk. 2	Wk. 1	Wk. 2	Wk. 1	Wk. 2
6	0	0	0	73	81	0·1	0·1	1·5	0·7	2	1	1	0
7	0	0	0	72	51	1·9	3·1	34·0	21·0	4	3	2	3
8	0	0	0	61	57	2·6	1·6	11·1	5·8	4	3	3	2
9	0	0	0	76	66	3·1	0·7	18·0	1·5	3	2	3	1
10	32	0	0	81	86	0·1	0	0	0	2	0	0	0
		Total		363	341	7·8	5·5	64·6	29·0	15	9	9	6
		Average		73	68	1·5	1·1	12·9	5·8	3	2	2	1
23	0	0	1	87	85	6·7	0·9	5·9	6·9	4	3	3	1
24	0	0	1	91	82	3·3	1·7	3·3	3·5	2	2	2	1
25	0	0	2	86	69	4·9	0·7	7·3	0·5	4	3	3	2
26	32	0	0	98	91	4·2	0	0·4	0	2	1	1	0
27	64	0	1	72	58	5·0	2·7	14·4	11·4	3	3	3	3
28	64	0	1	87	82	6·3	0·9	6·8	8·6	3	2	3	2
29	64	0	1	64	66	5·7	6·3	8·0	3·2	3	3	4	2
30	128	0	0	39	21	7·2	3·1	10·2	0·6	3	3	4	4
31	128	0	1	32	17	10·2	4·7	41·0	6·3	4	3	2	2
32	128	0	2	68	58	7·5	7·1	5·0	7·2	4	4	3	3
33	128	0	2	71	58	12·5	5·9	13·7	10·3	4	4	3	3
34	128	0	2	83	82	7·4	1·2	8·4	5·2	3	2	3	0
		Total		878	769	80·9	35·2	124·4	63·7	39	33	34	23
		Average		73	64	6·7	2·9	10·4	5·3	3	3	3	2
40	0	0	1	18	7	7·4	3·1	91·0	69·1	4	3	5	5
41	0	0	1	64	60	5·4	1·7	78·5	22·5	4	2	2	2
42	0	0	4	14	16	12·0	7·7	82·1	99·0	4	3	4	2
43	0	0	4	53	59	6·9	1·7	68·5	22·7	4	2	3	3
44	32	0	4	84	82	2·9	0·9	5·9	0·2	4	2	3	2
		Total		233	224	34·6	15·1	326·0	213·5	20	12	17	14
		Average		47	45	6·9	3·0	65·0	42·7	4	2	3	3
		Grand total		1474		123·3		515·0		74		60	
		Average		67		5·6		23·4		3·4		2·7	
		Grand total			1334		55·8		306·2		54		43
		Average			61		2·5		13·9		2·5		2·0

week, and in two of them counts at this time were negative. These findings therefore confirmed the association between staphylococcal hyaluronidase inhibitor and some protection against *Str. agalactiae*, and it seemed possible that vaccination with *Str. agalactiae* and the possession of staphylococcal hyaluronidase inhibitor in the serum might have some synergistic action in protecting against an intramamary test dose. Examination of the figures in Table 2 showed that if the results following the test dose of 10^5 organisms were neglected (as they were not homogeneous with the results following the 10^7 and 10^8 test doses), and if the measurement of protection were taken as the absence of *Str. agalactiae* from the milk in the second week, then no synergistic action could be demonstrated.

The figures in Table 2 also suggest the possibility of a loose correlation between protection against the test dose (measured by the absence of *Str. agalactiae* from the milk in the second week) and high serum titres of either streptococcal hyaluronidase inhibitor, or of A 100 agglutinins, at the time of challenge. Nevertheless, as in goats, these serum tires are not an accurate guide to the resistance of the animal to infection.

Alterations in milk yield. In earlier experiments with goats (Pattison and Holman, 1951) it was found that although all animals were susceptible to intramammary infection with *Str. agalactiae* the mastitis produced varied greatly in extent and intensity from animal to animal; in some cases the fall in milk yield from the infected quarter was slight, but in others the yield was reduced to nil. Howell, *et al.* (1954[a]) described "transient," "persistent," and "prolonged" experimental infections with *Str. agalactiae* in the cow and drew attention to the effect on milk yield of these different reactions and to the unreliability of alteration in milk yield of the infected quarter as a true measurement of infection.

In view of these earlier observations it was not expected that variations in yields from the infected quarters between vaccinated and control groups of cows would be easily interpreted, and no real difference between the groups over the second week of infection could be demonstrated even when the absolute yields for each cow were examined. However, as mentioned above, there was a significant tendency for vaccinated cows to show an increased yield in the second week of infection, compared with the first week, whereas control cows continued to lose milk in the second week.

Other points of interest regarding milk yields are shown in Table 3 which contains the average daily yields in lb. per cow for vaccinated and control animals exposed to each of the three test doses. Pre-infection yields were calculated from daily milk recordings made during the 11 weeks before inoculation of the intramammary test dose, and the predicted yield for the second week after challenge was determined by assuming that the trend of this pre-infection

TABLE 3

AVERAGE DAILY MILK YIELD IN LBS
ALL COWS ARRANGED ACCORDING TO TEST DOSE

Infecting dose	10^5	10^7	10^8
Total yield	*10 cows*	*24 cows*	*10 cows*
Pre-infection yield	17·52	25·92	26·37
Predicted yield for 2nd week	16·66	25·29	23·86
Actual yield for 2nd week	15·26	22·55	22·69
Loss due to infection	1·40	2·74	1·17
Loss shown as percentage of predicted yield	8%	11%	5%
Regression Yield/Loss	0·95	1·07	0·99
Yield from infected quarter			
Pre-infection yield	4·92	8·40	8·11
Predicted yield for 2nd week	4·88	8·20	7·34
Actual yield for 2nd week	3·86	5·79	4·33
Loss due to infection	1·02	2·41	3·01
Loss shown as a percentage of predicted yield	21%	29%	41%
Regression Yield/Loss	0·84	0·76	0·82

yield would have continued in a straight line had it not been influenced by the test dose. The loss attributable to infection was, therefore, the predicted yield for the second week minus the actual yield.

Examination of the figures in Table 3 showed an obvious difference between the percentage milk loss from the infected quarter when compared with the loss from the whole udder. There was a correlation between the loss from the quarter and the size of the infecting dose, so that the 10^5 dose caused a loss of approximately 1 lb. per day, the 10^7 dose 2 lb., and the 10^8 dose 3 lb. With the 10^5 dose the loss from the udder as a whole was only slightly greater than the loss from the infected quarter, and with the 10^8 dose the loss from the whole udder was actually less than the loss from the single infected quarter. This could only be explained by assuming a compensatory increase in yield from the non-infected quarters, and this assumption was confirmed by plotting the yields of the non-infected quarters of several cows taken at random.

Another point of interest was a correlation between the loss of milk attributable to infection and the total yield of the udder before infection. This was investigated by calculating for the cows in each dose group the regression between the pre-infection yields and the yields in the second week after infection, and from the figures in Table 3 it can be seen that the regression in each case was approximately one. Therefore, the absolute loss of milk from the whole udder due to infection was the same in all cows irrespective of individual variation in total yield before infection. The regression figures for the loss from infected quarters (Table 3), however, were

significantly different and averaged 0.81. Therefore, the high-yielding quarter lost more milk as a result of infection than did the lower-yielding quarter.

Bacteriologically Negative Milk samples obtained from Cows harbouring Str. agalactiae.

Howell, *et al.* (1954[a]) reported previously that bacteriologically negative milk samples taken twice daily over four consecutive days indicated that a cow had freed itself from infection. This opinion must be modified in the light of more recent experiments, as we have now encountered two animals that had negative milk samples twice daily for four-and-a-half and five days respectively followed by positive samples and a typical clinical crisis. We have no reason to believe that reapperarance of streptococci in the milk after the negative period was due to re-infection, because in none of the many experiments with goats and cows that we have recorded has a non-inoculated quarter become infected, and because the organism obtained after the negative period has always been of the same serological type as that inoculated.

Duration of Immunity

It has been shown above that there was clear evidence of resistance to an intramammary test dose of 10^5 living *Str. agalactiae* in four out of five vaccinated cows when compared with five control animals (Table 2). It was therefore decided to examine the duration of immunity in these five animals by exposing them to a second challenge dose in their next lactation. They received no further vaccinations.

The second test dose was again of 10^5 living strain A 100 and was injected into a different quarter from that used on the first occasion. Sixteen months separated the second test dose from the first, and five new control cows in a comparable stage of lactation received the same inoculum into one quarter of the udder.

The results of this second exposure are given in Table 4. Comparison of the figures in Tables 2 and 4 show that the serum titres of streptococcal hyaluronidase inhibitor and of A 100 agglutinins had declined markedly since the time of the first test dose. The results in Table 4 show that the immunity previously demonstrated in cows 1 to 4 was still clearly present. Cow 5 that became infected after the first dose (Table 2) was again infected after the second test dose (Table 4), and comparison of this animal with the five controls showed no significant difference for any measurement. However, comparison of the vaccinated group as a whole with the control group showed an obvious difference in favour of vaccination. While it is realised that the effect of the first challenge may have acted as a secondary immunising stimulus we feel that such an effect would not be very great because in cows 1 to 4 we were

TABLE 4

DURATION OF IMMUNITY

RETEST IN THE SUCCEEDING LACTATION (16 MONTHS LATER) OF COWS NOS. 1 TO 5 BY A SECOND TEST DOSE OF 10^5 A.100 INJECTED INTO A DIFFERENT QUARTER; RECORDED AS DAILY AVERAGES FOR THE FIRST AND SECOND WEEKS AFTER INFECTION

	Reciprocals of final pre-infection blood titres			*Results for milk from infected quarter*									
				*% Milk yield****		*Neutrophil count 10^6/ml.*		*A.100 count 10^3/ml.*		*Whiteside test units*		*Milk appearance units*	
Cow No.	*AH* Staph*	*AH* Str.*	*A.100 Agg.*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*	*Wk. 1*	*Wk. 2*
Vaccinated													
1	0	0	2	77	79	1·1	0·0	0·0	0·0	1	0	0	0
2	0	0	4	86	91	1·6	0·6	0·2	0·0	2	2	0	0
3	0	0	4	77	75	2·3	0·4	0·0	0·0	2	2	0	0
4	0	0	2	82	78	1·2	0·1	0·0	0·0	2	1	0	0
5	0	0	0	74	69	6·8	2·8	19·8	0·8	4	2	3	2
	Total		..	396	392	13·0	3·9	20·0	0·8	11	7	3	2
	Average		..	79	78	2·6	0·8	4·0	0·2	2	1	1	0
Controls													
45	0	0	0	71	66	7·4	9·6	16·0	11·5	3	4	3	4
46	0	0	0	36	55	7·6	0·6	7·7	0·0	3	2	3	1
47	0	0	0	97	60	5·5	5·8	9·9	17·2	4	3	3	4
48	0	0	0	62	53	3·1	1·1	17·0	4·0	3	2	3	1
49	0	0	0	67	55	2·0	1·7	18·3	15·4	2	3	1	1
	Total		..	333	289	25·6	18·8	68·9	48·1	15	14	13	12
	Average		..	67	58	5·1	3·8	13·8	9·6	3	3	3	2

*Anti-hyaluronidase

**Milk yield as % of pre-infection yield.

unable to recover viable organisms from the milk at the time of the first challenge even five hours after inoculation and in cow 5 in which there was a transient mastitis in week 1 and in which, possibly, some influence on the level of immunity could have been exerted the mastitis provoked at the time of the second challenge was clearly more intense (Table 4). It was concluded therefore that the immunity that was present in these cows had persisted for 16 months.

DISCUSSION

The main purpose of this work was to see whether the vaccination of cows with formolised whole cultures of *Str. agalactiae* would produce a resistance to an intramammary test dose of the same organism. This had been previously demonstrated in goats and the results recorded in this paper show that it also applies to the cow.

Furthermore there is some evidence that the immunity in the cow may persist for at least 16 months. The practical implication of this conclusion is that vaccination may have a place in the control of streptococcal mastitis, but further work is necessary to determine the conditions under which such a procedure might be economically valid. The favourable results in our experiments on the value of vaccination in streptococcal mastitis have been discussed previously (Holman, *et al.*, 1952) in relation to the findings of other authors, and we have no reason to modify the conclusion then reached that "this field of enquiry might well yield results of practical importance." Since vaccination has been shown to produce some protection against *Str. agalactiae* mastitis, it seems that problems associated with immunity against staphylococci, corynebacteria, and other mastitis organisms might well repay serious consideration.

The true meaning of the relationship between the presence of staphylococcal hyaluronidase inhibitors in the peripheral blood of cows and resistance to *Str. agalactiae* mastitis is still obscure, but there is now additional evidence to that presented by Howell, *et al.* (1954[b]) that these antibodies are associated with the resistance of the udder to this infection. It is pertinent, therefore, to record that regular examinations of cow sera over periods of months have disclosed that the staphylococcal hyaluronidase inhibitor antibody can develop in some animals without any clinically obvious evidence of staphylococcal disease.

Many authors have paid attention to various factors that may be involved in determining the susceptibility of the udder to mastitits. The anatomical structure of the teat and teat sphincter, the bacteriostatic properties of milk, the effect of milking technique, the activity of a virus, and so on, have at various times been described as predisposing causes of mastitis. Some, or all, of these factors may indeed be involved, but it seems to us necessary to draw attention to the fact that the immunological status of the cow must be considered as playing a major part in determining whether mastitis shall become clinically manifest. All our evidence points to the fact that circulating antibodies are of great importance in mastitis. Sometimes these antibodies are easily detectable, as in the case of naturally occurring staphylococcal hyaluronidase inhibitors, or of streptococcal hyaluronidase inhibitors or agglutinins produced by vaccination, and their presence can be related to the incidence of the clinical disease. Sometimes, however, antibodies may only be detectable in minimal amounts, or not at all, and yet evidence that they modify the course of the disease is impressive. It would appear however, that even when these antibodies have fallen below a level not detectable by *in vitro* methods, some resistance still exists, as shown in the case of the five animals re-exposed to infection 16 months after the first test dose.

CONCLUSIONS

Forty-four cows were divided into two equal and comparable groups according to their serum titres of detectable inhibitors to staphylococcal hyaluronidase. The animals in one group were given six subcutaneous vaccinations with .10 ml. amounts of formolised 18-hour broth cultures of *Str. agalactiae* strain A 100. Control animals received similar doses of formolised broth.

Animals in both groups were exposed to a single intramammary test dose of living *Str. agalactiae* strain A 100 injected into one quarter. The doses used were—five pairs of cows 10^5 organisms, 12 pairs 10^7 organisms, five pairs 10^8 organisms.

Analysis of the results obtained for the second week after infection showed that the vaccinated animals became free of infection more frequently and had lower neutrophil counts in the milk than control animals. An analysis of covariance showed no significant difference in milk yield from the infected quarters between the groups, but when the yields of the second week were compared with the first week the vaccinated cows tended to show an increase in yield and the controls a further drop in yield. The Whiteside test and the index for the macroscopic appearance of the milk showed little difference between the groups.

A comparison of the measurements made on animals challenged with the various test doses showed that the group that received 10^5 living organisms was not homogeneous with the 10^7 and 10^8 groups. In the case of the 10^5 group *Str. agalactiae* was not recovered on any occasion from four of the five vaccinated cows.

The five vaccinated animals exposed to the 10^5 test dose were exposed to a second test dose in their next lactation (16 months later). Four of the five still showed a high level of resistance.

REFERENCES

Holman, H. H., Pattison, I. H., and Gordon, W. S. (1952). *J. comp. Path.*, **62**, 196.
Howell, D. G. (1954). *J. Path. Bact.*, **68**, 287.
Howell, D. G., Pattison, I. H. Holman, H. H., and Smith, I. M. (1954[a]). *J. comp. Path.*, **64**, 335; (1954[b]). *Ibid.*, 351.
Mainland, D. (1948). *Canad. J. Res., E.*, **26**, 1.
Pattison, I. H. (1951). *J. comp. Path.*, **61**. 71.
Pattison, I. H., and Holman, H. H. (1951). *Ibid.*, 26; (1953). *Ibid.*, **63**, 304.
Pattison, I. H., Matthews, P. R. J., and Howell, D. G. (1955). *J. Path. Bact.*, **69**, 51.

[*Received for publication, July 6th*, 1955]

THE PRODUCTION AND INHIBITION OF THE HAEMAGGLUTININ OF *Cl. welchii*

By

Nancy Wickham

Department of Veterinary Pathology, University of Liverpool.

INTRODUCTION

Cl. welchii produces haemagglutinins of the direct type, that is, suspensions of organisms or filtrates containing the active substance lead to agglutination of red blood cells (R.B.C.) and the reaction can be inhibited by antitoxic or antibacterial sera prepared against the *Cl. welchii* group.

Claims that the reaction was specific and was of value in the diagnosis of *Cl. welchii* infections and for the typing of strains within the Group were made by Gürtürk (1952). He was successful in demonstrating haemagglutination (HA) with the 20 laboratory strains which he tested and in inhibiting the haemagglutination produced by each type with appropriate dilutions of homologous antiserum.

Partial confirmation of this work was reported by Dafaalla and Soltys (1953). They demonstrated the presence of a haemagglutinating factor in *Cl. welchii* cultures, which was specific for the welchii group, but they found that it was only possible to distinguish Type A from Types B, C and D in this way as the overlap between Types B, C and D prevented any sharp differentation between these groups.

Katitch (1954) had more difficulty in demonstrating haemagglutination. He followed Gürtürk's technique, but, whereas Gürtürk, and Dafaalla and Soltys were using strains which had been maintained for some time on artificial media, Katitch used strains which were for the most part freshly isolated. Of the 40 strains which he tested, 35 were isolated from sheep and lambs dead of enterotoxaemia or lamb dysentery and only 6 of these caused haemagglutination. The other 5 strains were laboratory strains and they, too, were negative. Four of the haemagglutinating strains were Type B, two were Type C, but the haemagglutinin of both types was inhibited by both B and C antitoxins.

The HA reaction of *Cl. welchii* and its inhibition by antisera is very similar to that which occurs with the influenza group of viruses, where the HA inhibition test has become a simple and efficient diagnostic aid. In view of the fact that Katitch found such a small number of haemagglutinating strains among recently isolated cultures, it seemed doubtful if the reaction would be of value as a diagnostic aid. But if Gürtürk's claim that the inhibition of each toxigenic type was specific could be confirmed, then the haemagglutination inhibition test offered a very much simpler method of typing strains than the usual toxin-antitoxin neutralisation tests in mice. It was with this aim that further work on the problem was undertaken.

MATERIAL AND METHODS

Strains of Cl. welchii. Fourteen stock strains were used. They had been maintained for a number of years as dried cultures and subsequently subcultured in Robertson's meat broth. Of these strains four were Type A, three Type B, one Type C and six Type D. In addition, fresh strains were collected whenever suitable material was available. Among these fresh isolations were seven strains of Type A, one of Type C and six of Type D. They were isolated from sheep, pigs, calves and puppies dying with symptoms suggestive of *welchii* infections. All the 28 strains used were checked for purity, for their reaction in litmus milk, for the absence of digestion in cooked meat medium, and were typed by toxin-antitoxin neutralisation tests in mice.

Media. The media used for the production of haemagglutinins were blood agar, nutrient agar, Robertson's meat-piece broth and nutrient broth. Cultures on blood agar and nutrient agar were incubated in a McIntosh and Fildes jar. Robertson's meat broth and nutrient broth were freshly boiled, and cooled rapidly before inoculation, then incubated without further precautions for obtaining anaerobic conditions.

Red blood cell suspensions. Defibrinated sheep's blood was usually used. The cells were centrifuged, washed three times in normal saline and the packed cells resuspended in normal saline to give a 1 per cent. concentration. Fresh cells were obtained once weekly, washed and stored in the refrigerator, and suspensions made up as required. Cells from sheep, bovines, pigs, chickens and human A cells were all tested for susceptibility to *welchii* haemagglutinins.

Technique for the haemagglutination test. If the organisms were grown in liquid culture, the tests were usually made with whole cultures, though it was found that the supernatant from which the organisms had been removed by centrifugation, or filtration, gave essentially the same titre.

If the organisms were grown on solid media, they were washed off with normal saline and, after the suspensions had been centrifuged at 3,000 r.p.m. for 15 minutes, the supernatant was used as antigen. Doubling dilutions of the antigen were made in normal saline and an equal volume (0·2 ml.) of one per cent. R.B.C. suspension added, the tubes shaken and allowed to stand at room temperature until the cells had settled (from four to five hours). Results were assessed on the pattern of the sedimented cells (Salk, 1944). Washings from uninoculated but incubated agar plates and uninoculated broth were tested and found negative for HA.

Sera. Antitoxic sera, prepared in horses against the four main *welchii* strains, A, B. C and D, were obtained from Burroughs Wellcome Ltd. Two other antisera against A were also obtained from Burroughs Wellcome, one of these being the "Wellcome" gas gangrene antitoxin (*perfringens*) prepared for human use, and the other an antitoxin prepared against Type A for experimental purposes.

In addition, an antiserum against a known haemagglutinating strain of Type A was prepared in rabbits. The strain used, A^3, was selected because of its relatively high titre of haemagglutinins (1: 256) when grown on nutrient agar. After 24 hours growth in Robertson's meat broth the HA titre of the supernatant was 1:64; 0·4 per cent. formalin was added to the supernatant, which was incubated at 37°C. for three days, and then used for the inoculation of two rabbits. The injections were given intra-

venously in increasing doses daily for the first three days (0·5 cc., 0·75 cc. and 1·0 cc); the rabbits were rested for ten days and then given three daily injections of 1·0 cc. Ten days after the last injection, the animals were bled and the serum collected.

Serum inhibition tests. The antigen to be used was titrated for its HA capacity. The last tube showing agglutination was taken as the end-point and this dilution of antigen was said to contain one HA dose. For serum inhibition tests the antigen was then diluted so that 0·2 ml. contained three haemagglutinating doses.

Doubling dilutions of serum were made in saline, and an equal volume of antigen dilution added. For the most part, this mixture of serum and antigen was incubated at 37°C. for 60 minutes before adding an equal volume of one per cent. R.B.C. suspension to each tube. The tubes were then shaken and allowed to settle at room temperature. Controls of antigen plus R.B.C., and the lowest dilution of serum plus R.B.C., were always included. Though this was the technique usually followed, there was apparently no difference in the results when the three reagents were added one after the other without any period of incubation for neutralisation to occur.

RESULTS

Effect of Medium on Production of Haemagglutination

Strains producing HA did so to a varying degree whether the organisms were grown on blood agar, nutrient agar, Robertson's mea -piece broth or nutrient broth. The efficiency of the media in respect of HA production was in the order given. Titres obtained with one strain grown on different media were as follows :— Blood agar: 1:512; nutrient agar: 1:256; Robertson's meat broth: 1:64; nutrient broth: 1:16. For most experiments, nutrient agar and Robertson's meat broth were the chosen media. Nutrient agar was used in place of blood agar because of the remote possibility of cross reactions occurring between the serum from the washings from the plates and the R.B.C. used in the test—the blood in both cases usually being from sheep. Robertson's broth was used because it was a convenient medium which could be incubated aerobically.

The addition of one per cent. glucose to either Robertson's meat broth or nutrient broth resulted in a more prolific growth of organisms but a much reduced yield of haemagglutinin. The pH of the media appeared to have a marked effect on the amount of demonstrable HA produced by a culture. A pH of 7.0 was the optimum for haemagglutinin production. In more acid media, the HA titre was reduced. In rapidly growing glucose broth culture, where the pH was as low as 5.5, tests for HA were negative, but if, during the growth of the organisms, the pH of the culture was constantly adjusted to pH 7.6 by the addition of NaOH, then haemagglutination could be demonstrated. Another complication in glucose cultures was the increased production of haemolysin, which may have masked the production of HA. Normally, haemolysins were only troublesome with A and C strains but, in glucose cultures, all strains produced lysins to high titre.

Effect of R.B.C. Suspension

Cells from different individuals of the same species varied a little in their susceptibility to agglutination, but the differences were not considered of sufficient significance to invalidate the results of any test.

There was little to choose between cells of different species and all cells tested were agglutinated by representative strains. Sheep cells were used in preference to those of other species, because they were readily available and they were known to give no cross reactions with horse or rabbit sera which were to be used for the inhibition tests, (Dafaalla and Soltys, 1951).

TABLE 1

TITRES OBTAINED WITH THE CELLS OF DIFFERENT INDIVIDUALS AND DIFFERENT SPECIES. ONE STRAIN OF *WELCHII*, GROWN UNDER SIMILAR CONDITIONS.

Species	*1: 64*	*1: 128*	*1: 256*	*1: 512*
Sheep	2	12	2	—
Ox	1	4	1	—
Pig	—	3	3	—
Chicken	—	3	—	—
Human Gr. A ..	—	—	3	1

The figures represent the number of individual strains tested.

Strain Differences in the Production of Haemagglutinin

With many strains of *Cl. welchii*, HA occurred readily when saline suspensions of organisms, the supernatant from saline suspensions of organisms or the supernatant from Robertson's broth cultures were left in contact with suspensions of R.B.C. Some strains grown and tested under similar conditions gave much lower titres and other strains were negative. Titres varied from 0 to 1:256 or more. These variations in agglutinin capacity could not be correlated with the classification of types within the *welchii* family. Representatives from each of the four toxigenic types were among those which gave no HA, those which gave a low titre of HA and those which gave high titres.

The only consistent finding was that all negative strains were freshly isolated ones. Of the fourteen so tested, only one produced HA and then only to a titre of 1:32; moreover, strains which gave good titres of HA became negative after guinea-pig passage. With a view to testing whether variation in virulence might have a bearing on HA capacity, three strains (B, B^2 and C) were passaged through guinea-pigs. They proved highly virulent, the guinea-pigs dying within 24 hours and showing the typical post-mortem picture of *welchii* infection. In each case the organisms were recovered from

the thigh (the site of inoculation) and from the heart blood. These guinea-pig passaged strains differed from the parent cultures in that there was a complete absence of HA capacity, whereas the original strains gave titres of 1:256 or more. Twelve consecutive subcultures of the guinea-pig strains in Robertson's meat broth resulted in the production of haemagglutinin by one of them but only to a titre of 1:4. However, after approximately 18 months on artificial media. all had reverted spontaneously to HA production. All of the freshly isolated strains acquired the capacity for HA production after they had been subcultured for a variable period in Robertson's meat broth.

No differences in colony type, morphology or in the production of spores or capsules could be correlated with differences in HA production. There was a much greater production of haemolysins by freshly isolated or guinea-pig passaged strains when compared with laboratory strains, and this was at first thought to be masking the HA. However, on artificial media this greater production of lysins was lost much sooner than the capacity for producing HA appeared, and it was found that α and θ antitoxins could inhibit lysis but not HA.

The Specificity of the Haemagglutinin

The haemagglutinin produced by all strains was specific for *Cl. welchii*. It could be inhibited by *Cl. welchii* antiserum, but not by normal serum or by antiserum prepared against *Cl. oedematiens, Cl. septique, Cl. chauvoei, Cl. tetani* or *Cl. botulinum B*.

Within the *welchii* family, however, strains characterised by different toxin production could not be identified by this means. The HA of all strains could be inhibited to some degree by antitoxic sera of the four main groups. The variation in titre shown by the different sera appeared to have no relationship to the use of homologous or heterologous strains as antigens. Table 2 shows the reciprocal of the highest dilution of each type of sera which was effective in neutralising three haemagglutinating doses of representative strains of each type of *welchii*.

TABLE 2

INHIBITORY TITRE OF THE FOUR TYPES OF WELCHII ANTISERA. FOUR TYPES OF ORGANISMS

Serum	*Organisms*			
	A	*B*	*C*	*D*
A	16	4	4	4
B	2048	2048	2048	2048
C	512	512	512	1024
D	>4096	>4096	>4096	>4096

Only with the A antiserum was there any indication of preferential inhibition of the HA of the homologous strain. The other sera were consistent in their ability to prevent HA irrespective of the toxigenic type of the strain producing it.

In view of the earlier results showing differences in the titre of HA obtained with organisms of the same type, it was felt that the very low titre of inhibition obtained with A serum might be due to the fact that the strain used for its production gave poor HA. Other A sera were therefore obtained and tested. These were: (1) an antitoxic serum produced by Burroughs Wellcome for experimental purposes; (2) an antitoxic serum produced by Burroughs Wellcome for use in man; (3) an antiserum produced in rabbits against strain A3. The sera are designated Expt., Treatment and Anti-A3 respectively. Results are shown in Table 3. The figures show the reciprocal of the highest dilution of serum causing inhibition of HA (3 haemagglutinating doses). The tires obtained were much higher than those given by the original A serum used, and no preferential inhibition of the homologous strain was shown.

TABLE 3

TITRES OF HA INHIBITION OBTAINED WITH DIFFERENT ANTI-A SERA

Sera against strain	*Sera*		
	Expt.	*Treatment*	*Anti-A*[3]
A	1024	512	64
B	1024	256	32
C	1024	512	32
D	1024	512	64

The results given in Tables 2 and 3 suggested that the haemagglutinin of *Cl. welchii* was a group characteristic and not related to toxigenic types. Proof of this was difficult to obtain, mainly because the haemagglutinin was found to be soluble and easily washed off the organisms. This meant that absorption tests using bacteria were useless as the greater part of the HA was eluted into the fluid used for washing the organisms off the plates, and was therefore lost when the organisms were centrifuged and added as a heavy suspension to the serum; moreover, any still remaining on the cells was eluted into the serum and further complicated the assessment of results.

It was therefore decided to attempt neutralization of the haemagglutinin produced by each strain by each serum in such a way that the amount of serum used would exactly neutralize the amount of haemagglutinin added, and then by adding a known number of haemagglutinating doses of the homologous and heterologous strains

to see if any residual antibodies were left. To do this, each strain was grown on agar for 72 hours, washed off the plate with saline, centrifuged at 3,000 r.p.m. for 15 minutes, and the supernatant used as antigen. Equal volumes of this antigen and doubling dilutions of the serum under test were mixed and left at 37°C. for one hour; an equal volume of R.B.C. suspension was then added, the tubes shaken and allowed to settle at room temperature. The critical titre was somewhere between the last tube showing complete inhibition of HA and the first tube showing HA. A second titration of serum against antigen was made within this range at much closer intervals than before, and the last tube showing complete inhibition of HA regarded as the one which just neutralized the haemagglutinin contained in the volume of the supernatant used. The required amount of this dilution of serum was then mixed with an equal volume of the antigen and incubated for one hour for neutralization to occur. 0.1 ml. of the mixture was then added to 0.1 ml. of each strain A, B, C and D, respectively which had been previously titrated and diluted so that 0.1 ml contained three haemagglutinating doses. R.B.C. were then added, the tubes shaken and allowed to settle. Controls containing the critical dilution of serum and the absorbing antigen were also included.

The four antisera A, B, C and D were absorbed by representatives of each of the four strains A, B, C and D, and all were tested against homologous and heterologous strains. The result in every case was the same: agglutination occurred on adding the final three haemagglutinating doses, showing that absorption by both homologous and heterologous strains removed all the antihaemagglutinins from the sera.

DISCUSSION

The haemagglutination inhibition test proved to be of no value for the diagnosis of *welchii* infections or the typing of strains, firstly because few recently isolated strains were found to produce this phenomenon, and secondly because the haemagglutinin was found to be a group characteristic and not related to toxigenic types.

Haemagglutination was readily produced by many strains of *Cl. welchii*. Other strains grown and tested under the same conditions were negative in this respect. In our experience, negative strains were either recently isolated ones, or were laboratory strains which had been re-isolated after guinea-pig passage. Katitch (1954) not only failed to demonstrate HA with 29 of 35 freshly isolated strains but also with five laboratory strains. He does not state how long these latter strains had been kept on artificial media. From the observation that laboratory strains which gave good titres of HA became negative immediately on re-isolation after guinea-pig passage, it is obvious that a mutation must be involved, and one of the factors which produces this mutant character is long continued cultivation on laboratory media.

It has been shown by several authors (Livesay, 1933; McGaughey, 1933; Orr, Josephson, Baker and Reed, 1933; Stevens, 1935) that variants of *Cl. welchii* are readily produced in laboratory media, and that these may vary in toxicity and antigenic structure from the parent culture. Such variations, however, seem to have been always associated with definite variations in colony type. Orr, *et al.* (1933), for instance, describe S and R colony types, and state that "serial cultivation in various fluid media, particularly Robertson's meat broth, results in S-R and R-S variation; that R type is much less virulent than S, but there is some evidence that apparent killing action of R types may result from *in vivo* dissociation of R-S; and S types produce 10-20 times more toxin (haemotoxin) than R types."

Apart from the fact that we could not detect definite S and R colonies as those authors describe them, these statements are in keeping with our experience. A greater production of haemolysin was very obvious with recently isolated strains, and some dissociation must have occurred *in vivo* with the guinea-pig passaged strains, as these differed from the parent strain in HA capacity and production of haemolysin, although the colony type was not significantly altered. All colonies of a single strain appeared similar on blood agar, and no selection of colonies was made for isolation or subsequent subculture. It is possible that if this had been done, haemagglutinin production might have appeared earlier than it did naturally in strains which reverted to or acquired this capacity, and colonies varying in morphology might have been obtained.

The greater production of haemolysis by recently isolated strains was thought at first to be masking the haemagglutinin. That this was not the case was shown firstly by the fact that the production of excess lysins was lost earlier than the capacity to produce HA was acquired. For example, after twelve consecutive passages in Robertson's meat broth, lysins were no longer noticeable, but HA had not appeared, and, secondly, it was found taht α and θ antitoxin would inhibit lysis but not HA.

Whilst the inhibition of *welchii* HA was specific in that it could be inhibited only by *Cl. welchii* antiserum, antisera of the four main toxigenic types inhibited the reaction regardless of the type to which the strain producing the reaction belonged. The potency of the serum depended on the haemagglutinating capacity of the strain used for its production: this was shown by the different titres obtained with four different anti-A sera.

CONCLUSIONS

Haemagglutinins acting directly on the R.B.C of various species were produced fairly readily by many strains of *Cl. welchii*. The ability to produce HA appeared to be related to long continued cultivation on artificial media. The majority of recently isolated strains did not produce HA, and laboratory strains giving good

titres of HA failed to do so when re-isolated after guinea-pig passage. After a variable period on artificial media, however, there was a spontaneous reversion to HA production.

The haemagglutinin was specifically inhibited by *Cl. welchii* antisera. The effectiveness of any antiserum against the HA produced by the four toxigenic types A, B, C and D was almost equal; the haemagglutinin was therefore a group characteristic and not related to toxigenic types.

Because the HA was not produced by many freshly isolated strains and because it bore no relation to toxigenic types, the HA inhibition test was of no value as a diagnostic aid.

ACKNOWLEDGMENTS

The material reported in this paper formed part of a thesis for the degree of M.V.Sc. at the University of Liverpool.

My thanks are due to Professor R. E. Glover for the opportunity of working on this problem and for his advice throughout; also to the staff of the Veterinary Investigation Centre, Liverpool for access to material from which many of the fresh isolations of *Cl. welchii* were made.

REFERENCES

Dafaalla, E. N., and Soltys, M. A. (1951). *Brit. J. exp. Path.*, **32**, 510; (1953). *Nature*, **172**, 38.
Gürtürk, S. (1952). *Z. Hyg.*, **133**, 573.
Katitch, R. V. (1954). *Rev. d'Immunologie*, **18**, 199.
Livesay, H. R. (1933). *J. infect. Dis.*, **53**, 125.
McGaughey, C. A. (1933) *J. Path. Bact.*, **36**, 263.
Orr, J. H., Josephson, J. E., Baker, M. C., and Reed, G. B. (1933). *Canad. J. Res.*, **9**, 350.
Salk, J. E. (1944). *J. Immunol.*, **49**, 87.
Stevens, F. A. (1935). *J. infect. Dis.*, **57**, 275.

[*Received for publication, August 13th, 1955*]

THE RELATIONSHIP OF THE HAEMAGGLUTININ OF *Cl. welchii* TO OTHER SOLUBLE ANTIGENS OF THIS ORGANISM

By

Nancy Wickham

Department of Veterinary Pathology, University of Liverpool.

INTRODUCTION

While investigating the production of haemagglutination by *Cl. welchii*, it was observed that at least part of the factor responsible for this phenomenon was soluble. The titres of haemagglutination obtained with whole liquid cultures and the same cultures after centrifugation were essentially the same, and the titres were not significantly altered by filtration through Gradocol membranes. There was, therefore, a possibility that the haemagglutinin might be related to one of the other soluble antigens produced by this organism.

The haemagglutinin was produced by strains of the four *Cl. welchii* types A, B, C and D, so if any correlation was to be found, it must be with one of the antigens shared by the four toxigenic types. By reference to the table of Oakley and Warrack (1953), the possibilities appeared to be alpha and theta toxins, desoxyribonuclease, and perhaps the enzymes affecting red blood cells, for they have apparently been sought only in types A and B, and their presence in types C and D is uncertain.

Lecithinase, theta toxin and desoxyribonuclease were excluded from any participation in haemagglutination, but there seemed to be a definite relationship between the haemagglutinin, the enzyme destroying blood group substance A and the enzyme destroying the virus receptors on red cells, (R.D.E.). This paper is concerned with the enzymes destroying blood group substance A and the R.D.E.

MATERIALS AND METHODS

Strains of Cl. welchii *used.* These were representatives of the four main toxigenic types. The majority had been maintained for some time on artificial media, others were freshly isolated, and three were laboratory strains passaged through the guinea-pig. These guinea-pig passaged strains differed from the parent culture in that they no longer caused haemagglutination.

All cultures were grown for 48 hours in Robertson's meat piece broth. The media was freshly boiled and cooled before inoculation, then incubated without further precautions for obtaining anaerobic conditions.

Technique for the haemagglutination (HA) test. Robertson's meat broth cultures were centrifuged at 3,000 r.p.m. for 15 minutes, and the supernatants used as antigen. Doubling dilutions were made in 0.2 ml. of normal saline and 0.2 ml. of one per cent suspension of red blood cells (R.B.C., human group A or sheep cells) were added. The tubes were

shaken and allowed to settle at room temperature. When the controls had settled, the results were read according to the pattern of the sedimented cells. (Salk, 1944).

Serum inhibition tests. The sera used were Burroughs Wellcome Antitoxic sera. Doubling dilutions were made in saline, and an equal volume of antigen dilution containing three HA doses added. After one hour at 37°C., 0·2 ml. of one per cent suspension of R.B.C. was added, the tubes shaken and allowed to settle at room temperature.

Technique for the demonstration of the enzyme destroying group A substance. This was based on the work of Schiff (1939), Morgan (1946) and Stack and Morgan (1949) who showed that an enzyme produced by strains of *Cl. welchii* types A and B, destroyed the water soluble group specific substance A which is present in human saliva and in commercial peptone.

The supernatant from Robertson's meat piece broth was used as the source of group A substance and found to contain sufficient to inhibit the isoagglutination of human A cells by anti-A serum. Anti-A serum was titrated for its isoagglutinin titre. Doubling dilutions of the serum were prepared in 0·2 ml. saline, 0.2 ml. of saline added, and 0 2 ml. of one per cent suspension of R.B.C. The cells were allowed to settle at room temperature, and agglutination was read as before by the pattern of the sedimented cells. The titre of agglutination so obtained was compared with the titre obtained with the same cells and serum, when 0.2 ml. of supernatant from uninoculated Robertson's meat broth was substituted for saline, and with the titre obtained when 0·2 cc. of supernatant (or filtrate) from 48 hour Robertson's broth cultures of the organism to be tested was substituted for saline.

Inhibition of the enzyme destroying blood group A substance by Cl. welchii *antisera.* Filtrates from representatives of the four strains A, B, C and D were incubated with broth, and the fact confirmed that such incubation would destroy the A substance. Similar filtrates were then mixed with the four types of antitoxic sera and the sera titrated for their power to prevent destruction of A substance. Equal volumes of filtrate and dilutions of the appropriate serum were mixed and allowed to stand for two hours at 37°C. An equal volume (0·2 ml.) of broth was then added and the mixture incubated overnight; in the morning, 0 2 ml. of anti-A serum was added to 0·2 ml. of the mixture, and after 30 minutes 0·2 cc. of one per cent suspension A cells completed the test. Controls contained: (1) saline in place of the antitoxic serum, i.e. saline and filtrate, to check the activity of the filtrate; and (2) saline in place of both antitoxic serum and filtrate to check the activity of the broth at the dilution used in the test and the effect of overnight incubation.

Technique for demonstrating the modification of the R.B.C. The test used was based on the "quantitative" titration described by McCrea (1947) and modified by Stone (1947). Two strains of virus were used, Newcastle disease virus (N.D.V.) and the influenza strain, PR.8. These were inoculated into embryonated eggs on the tenth day of incubation, using the allantoic route and the technique described by Beveridge and Burnet (1946). After 48 hours further incubation the allantoic fluid was pipetted off and its HA titre determined. Doubling dilutions of *Cl. welchii* supernatants were prepared in 0·2 ml. saline, 0·2 ml. of one per cent washed R.B.C. suspension (human A cells) added to each tube and the series incubated in a water-bath for two hours (for N.D.V.) or four hours (PR.8)

at 37°C. After this time, the cells had sedimented completely and the supernatants were removed and discarded. The cells were then mixed with 0·2 ml. of normal saline plus 0·2 ml. of the appropriate virus dilution containing 10HA doses, and allowed to settle at room temperature.

The inhibitory action of Cl. welchii *antisera on the R.D.E.* The four sera, A, B, C and D, were diluted in normal saline. 0·2 ml. of serum dilution plus 0·2 ml. of the filtrate to be tested were incubated for one hour at 37°C.: 0·2 cc. of one per cent suspension of R.B.C. was then added to each tube and incubation continued for four hours. The supernatants were removed from the deposited cells, and 0·2ml. of saline or 0·2 ml. of N.D.V. or PR. 8 containing 10 HA doses were added. Agglutination was read after the cells had settled at room temperature. Control tubes, containing 0·2 ml. of filtrate plus 0.2 ml. saline in place of antisera, were incubated at the same time, and R.B.C. and virus added as before.

RESULTS

The Haemagglutinin and the Enzyme Destroying Blood Group Substance A

The production of the enzyme. Twenty strains of *Cl. welchii* were tested in parallel for their HA capacity and for their capacity to destroy the A substance in broth. The production of the enzyme destroying group A substance was common to the four toxigenic types of *Cl. welchii* and was correlated with the production of the haemagglutinin. The results are given in Table 1. Three strains D^9, D^7 and A^6 did not cause HA but allowed isoagglutination to

TABLE 1

THE TITRE OF AGGLUTINATION OF ANTI-A SERUM AGAINST A CELLS. THE EFFECT OF UNINOCULATED BROTH AND BROTH IN WHICH *CL. WELCHII* HAD GROWN

Isoagglutination titre	*In the presence of*	*HA titre of culture*
1: 2560	Saline	
–ve	Broth	
1: 5120	Strain A	1: 32
1: 5120	„ A^3	1: 32
1: 40	„ A^6	–ve
–ve	„ A^7	–ve
–ve	„ A^9	–ve
–ve	„ A^{10}	–ve
1: 5120	„ B	1: 16
–ve	„ B g.pig	–ve
1: 5120	„ Bm	1: 16
1: 5120	„ Bm g.pig	1: 512
1: 5120	„ Bc	1: 16
1: 5120	„ C	1: 256
–ve	„ C g.pig	–ve
–ve	„ C^1	–ve
1: 5120	„ D	1: 128
–ve	„ D^1	–ve
1: 5120	„ D^4	1: 16
1: 160	„ D^7	–ve
–ve	„ D^8	–ve
1: 10	„ D^9	–ve

occur to the very low titres of 1:10, 1:160 and 1:40 respectively, so that some of the A substance normally present in broth must have been destroyed. This observation suggested the possibility that all strains might produce the factor destroying blood group A substance in varying degree, and that only when it was present in excess was there any surplus to produce HA. It was thought that if the amount of peptone, and consequently the amount of blood group A substance, in the media could be reduced, then HA might be demonstrable to a higher titre in those strains ordinarily showing HA, and HA might appear with strains not usually showing this phenomenon. This, however, did not occur in the modified media used. Broth and agar containing only meat extract without added peptone were tried, as well as broth containing peptone from soya bean in place of peptone of animal origin. When HA strains of the organism were grown in such media, neither HA nor destruction of A substance could be demonstrated.

Panagglutination. When supernatants from cultures of *Cl. welchii*—producing haemagglutination were mixed with group A cells and anti-A serum, the titre of agglutination obtained was far in excess of the original isoagglutinin titre, and in excess of what one would expect from the combined effect of isoagglutination plus *Cl. welchii* agglutination. This phenomenon was shown to be due to panagglutination. Not only did these filtrates render A cells more susceptible to anti-A serum, but A cells became susceptible also to anti-B serum to the same high titre, and sheep cells showed greatly increased susceptibility to anti-A serum. (Table 2).

TABLE 2

PANAGGLUTINATION WITH SUPERNATANT FLUIDS

Mixture	*Titre*
A cells+anti-A serum+saline	1: 2560
A cells+anti-A serum+*welchii* A^3	1: 20,480
A cells+anti-A serum+*welchii* B	1: 20,480
A cells+anti-A serum+*welchii* D	1: 20,480
A cells+anti-B serum+saline	–ve
A cells+anti-B serum+*welchii* A^3	1: 20,480
Sheep cells+anti-A serum+saline	1: 40
Sheep cells+anti-A serum+*welchii* A^3 ..	1: 20,480

This increased susceptibility of the cells occurred when they were in contact with the filtrate and antiserum during the time taken for settling to occur, as in the usual procedure for the HA test; or when

they were added to the filtrate, left for ten minutes or more at room temperature, then centrifuged, washed with saline, and made up to a one per cent. suspension. In contrast, broths from strains of *Cl. welchii,* which did not produce HA, not only failed to increase the susceptibility of the R.B.C., but like uninoculated broth, inhibited isoagglutination. Active filtrates diluted beyond the HA titre did not increase the susceptibility of the cells to agglutination.

The panagglutinating effect was produced in addition to the destruction of A substance, for all cultures active in this respect produced HA, and HA was shown to be inhibited by the presence of A substance in the form of uninoculated broth.

D^4 filtrate + saline + sheep cells : HA 1:32
D^4 ,, + broth + sheep cells : HA —

Inhibition of the enzyme by antisera. The action of *Cl. welchii* filtrates on R.B.C. could be inhibited by *Cl. welchii* antiserum of the four toxigenic types. It was found that at least ten times the haemagglutinating dose was required to destroy the amount of A substance necessary to inhibit the action of anti-A serum against A cells. Table 4 shows the inhibitory titre of the sera described in Table 3 against ten HA doses and against three HA doses of haemagglutinin.

TABLE 3

INHIBITION OF THE ENZYME DESTROYING BLOOD GROUP SUBSTANCE A BY *CL. WELCHII* ANTISERA

Serum	*Serum dilution*				
	Undiluted	*1: 100*	*1: 500*	*1: 2500*	*1: 5000*
Welchii Serum anti-A	+	—	—	—	—
,, ,, ,, B	+	+	+	—	—
,, ,, ,, C	+	+	—	—	—
,, ,, ,, D	+	+	+	+	+

— = no inhibition of enzyme action against A substance.
+ = inhibition ,, ,, ,, ,, ,, ,,

TABLE 4

INHIBITION OF THE HAEMAGGLUTININ BY *CL. WELCHII* ANTISERA

Serum	*Titre of serum against ten HA doses*	*Titre of serum against three HA doses*
Welchii anti-A ..	undil.	1: 4
,, ,, B ..	1: 1000	1: 2048
,, ,, C ..	1: 100	1: 512
,, ,, D ..	>1: 5000	>1: 5000

Physical properties of the enzyme destroying blood group A substance. Schiff (1939) showed that the A destroying enzyme was affected by acid and that media containing one per cent glucose (pH 6.0) was sufficiently acid to destroy it. He also showed that it was heat labile and could be precipitated by acetone. These properties apply also to the haemagglutinin. It has already been shown (Wickham, 1955) that the haemagglutinin is susceptible to acid and that HA is not demonstrable if strains are grown in media containing glucose. Filtrates containing the HA were tested for their HA capacity before and after heating to 56°C for 30 to 60 minutes. It was found that the activity of the haemagglutinin was greatly reduced after 30 minutes at this temperature and completely destroyed after one hour (Table 5).

TABLE 5

SHOWING THE DESTRUCTION OF HA BY HEATING

	Original titre	*After heating at 56°C. for 30 mins.*	*After heating at 56°C. for 60 mins.*
Strain B	1:32	1:2	–ve
„ D^4	1:32	1:4	–ve
„ A^3	1:128	1:64	–ve

Acetone precipitation was tried on a supernatant known to cause HA. The capacity of the washed redissolved precipitate to cause HA, and to destroy the A substance in broth, was compared with that of the original filtrate. The precipitate showed both activities but little increase in titre over that of the original filtrate. For example, a filtrate of strain A produced HA to a titre of 1:32 and the redissolved precipitate to a titre of 1:64. The filtrate destroyed the A substance in broth when diluted 1:2 and the acetone precipitated material was active when diluted 1:4. It was clear, however, that both substances were precipitated by acetone.

The Haemagglutinin and the R.D.E

Strains producing the R.D.E. Evidence was obtained that the R.D.E. was produced only by those strains producing HA. The titre for the R.D.E., however, was in general slightly higher than the titre of HA. Table 6 shows the results of quantitative titrations carried out with N.D.V. and PR.8 virus. No HA occurred during incubation of cells and *Cl. welchii* filtrate; the cells settled out in the discrete central button of a negative test, but if the tubes were then shaken and allowed to resettle at room temperature, agglutination occurred to the same titre as controls left from the beginning of the experiment at room temperature. With some strains, haemolysis occurred during incubation, but it was found that this could be controlled by using citrate saline in place of normal saline as the diluent (McCrea, 1947).

TABLE 6

THE EFFECT OF HA AND NON-HA STRAINS OF *CL. WELCHII* ON N.D.V. AND PR.8 HA

Strain of welchii	*HA titre*	*Dilution of welchii filtrate* 1:2	1:4	1:8	1:16	1:32	1:64	1:128	*Control*
A^3	1:32	—	—	—	—	—	—	—	NDV +++
		—	—	—	—	—	—	+++	PR8 +++
B	1:32	—	—	—	—	—	—	—	NDV +++
		—	—	—	—	—	—	+++	PR8 +++
B g.pig	–ve	+++	+++	+++	+++	+++	+++	+++	NDV +++
		+++	+++	+++	+++	+++	+++	+++	PR8 +++
BM	1:16	—	—	—	—	—	+	+++	NDV +++
		—	—	—	—	—	+++	+++	PR8 +++
D^4	1:32	—	—	—	—	—	—	—	NDV +++
		—	—	—	—	—	+++	+++	PR8 +++
D^6	–ve	+++	+++	+++	+++	+++	+++	+++	NDV +++
		+++	+++	+++	+++	+++	+++	+++	PR8 +++
A^6	1:64	—	—	—	—	—	—	—	NDV +++
		—	—	—	—	—	—	+	PR8 +++
A^{10}	–ve	+++	+++	+++	+++	+++	+++	+++	NDV +++
		+++	+++	+++	+++	+++	+++	+++	PR8 +++
D^7	1:64	—	—	—	—	—	—	+	NDV +++
		—	—	—	—	—	—	+++	PR8 +++
D^8	1:16	—	—	—	—	—	—	—	NDV +++
		—	—	—	—	—	—	++	PR8 +++

Inhibition of the R.D.E. The activity of the filtrates against the R.B.C. could be inhibited by anti-*welchii* sera of all types, and the sera showed practically the same titre against this enzyme as against the HA factor. Table 7 shows the results obtained using *Cl. welchii* filtrate of strain A^3 (three HA doses) and virus PR.8.

TABLE 7

INHIBITION OF R.D.E. BY *CL. WELCHII* ANTISERA

Serum	*Dilution* 1:10	100	500	1000	2000	5000	10000
A	+++	—	—	—	—	—	—
B	+++	+++	+++	+++	—	—	—
C	+++	+++	+++	—	—	—	—
D	+++	+++	+++	+++	+++	+ ++	+++

Control filtrate, saline and P.R.8 — PR. 8 control +++

Elution of the HA from the R.B.C. One of the main characteristics of the R.D.E. as described by McCrea (1947) is its capacity for adsorption and elution from the R.B.C. The HA was tested for this

capacity. The method used was that of McCrea (1947), modified to allow ten minutes adsorption. The results are shown in Table 8.

TABLE 8

ELUTION OF HA

HA titres	*Strains*			
	A[3]	*B*	*D*[4]	*A*[4]
Original supernatant	1:64	1:16	1:32	1:16
Supernatant after ten minutes adsorption by R.B.C.	–ve	–ve	–ve	–ve
Citrate-saline supernatant after one hour at 37°C.	1:4	1:4	1:8	1:2
Citrate-saline supernatant after two hours at 37°C.	1:32	1:8	1:8	1:4

Elution of the HA factor occurred though the recovery of the enzyme was in no way complete.

DISCUSSION

From the results of these experiments there is a strong suggestion that the haemagglutinin, the enzyme destroying blood group substance A, and the R.D.E. are closely related if not identical.

The enzyme destroying blood group substance A is described by Schiff (1939) as heat labile, inactivated by an acid pH and precipitated by acetone. These criteria apply also to the HA factor, and the power to destroy blood group substance A to any significant degree was found to be confined to strains which produced HA. Moreover, antisera active against the HA showed a parallel activity against the enzyme destroying blood group substance A. It is possible, therefore, that these two substances are identical and that haemagglutination occurs because of the destruction of blood group A substance on the R.B.C. If this hypothesis were correct, then other organisms and enzymes capable of destroying the A substance, such as those described by Morgan (Landsteiner and Chase, 1935), Sickles and Shaw (Chase, 1938), Chase, (1938) and Schiff, (1939), should also cause haemagglutination and, conversely, organisms causing direct agglutination of R.B.C. should be capable also of destroying the A substance.

McCrea (1947) suggested that the enzyme destroying Group A substance might be the same as the R.D.E. which had been obtained from *Cl. welchii* Type A as well as from the cholera vibrio. It was shown by Burnet, McCrea and Stone (1946) that these enzymes modified the virus receptors of red cells in the same order as the viruses of mumps, Newcastle disease, influenza A, influenza B and swine influenza, and they considered them identical with the virus

enzymes. Filtrates from both these organisms have since been shown to cause direct agglutination of R.B.C., the cholera vibrio (Burnet, McCrea and Stone, 1946) and *Cl. welchii* (Gürtürk, 1952; Dafaalla and Soltys, 1953; Katitch, 1954). The work reported here showed that the production of this receptor-destroying enzyme is not confined to Type A strains of *Cl. welchii;* strains of Types B, C and D also produced it and in all cases its production appeared to be correlated with the enzyme producing HA.

All these enzymes show the capacity to modify R.B.C. so that they are agglutinated by sera other than their specific group antisera. Such "panagglutination" was first demonstrated by Thomsen (1926), and Freidenreich (1928) showed that the increased agglutinability of the R.B.C. was caused by a bacterial enzyme. Burnet, *et al.* (1946) showed that the change produced on the R.B.C. by the viruses of the mumps-influenza group was similar to the "panagglutinability" described by Thomsen, and that partially purified preparations of *Cl. welchii* toxin, type A, and filtrates of young cultures of cholera vibrio had the same effect. We have shown that strains of all types of *Cl. welchii,* provided that they are haemagglutinating strains, produce panagglutination. It seems, therefore, that the two phenomena, panagglutination and haemagglutination, are brought about by the same mechanism; possibly by action of the enzyme concerned on the group specific substances of the red cell.

The substrate or receptor substance for the enzyme action has not been identified. De Burgh, Pen-Chung, Howe and Boranick (1948) prepared an inhibitory substance from human and sheep R.B.C. which they believed to be identical with the receptors and this was characterised as a polysaccharide or complex protein polysaccharide. Other inhibitor substances believed to be analogous with the receptor substance have been found in normal serum (Hirst, 1948) tissue cells (Friedewald, Muller and Whatley, 1947) and various body fluids (Burnet, McCrea and Anderson, 1947). Burnet, *et al.* (1947) found that fluid from a pseudomucinous cyst and blood group O substance inhibited virus agglutination and that this inhibition was reduced by incubating virus and O substance together. They have suggested that the substrate for the action of the virus enzyme is the mucin responsible for A B O blood group specificity. Evidence in favour of this hypothesis is provided by Green and Wooley (1947) who reported that blood group substance A inhibited influenza HA, and also by the distribution and chemical nature of these substances, which are very similar to those of the inhibitors and therefore of the receptors for virus HA. In addition, we have found that blood group A substance inhibits *Cl. welchii* haemagglutination, and that production of haemagglutinin by this organism is correlated with the production of the enzyme which destroys blood group substance A.

There are two other enzymes produced by *Cl. welchii* which have an effect on blood group substances. That which destroys blood

group substance H is heat stable (Morgan, 1947) and must therefore be a separate entity from that destroying blood group substance A. That which destroys Group B substance is also thought to be separate (Schiff, 1939; Stack and Morgan, 1949) although it is indistinguishable from the A destroying enzyme except in its group specificity. The group B enzyme has not been investigated for its haemagglutinin activity.

Although the various blood group substances can be sharply defined by appropriate antisera, those which have been isolated do not show any marked chemical differences. It has therefore been suggested by Boyd and Shapleigh (1954) that the structural differences between them which are responsible for their specificity may be relatively minor. In this connection, it may be significant that the O and H substances have long been confused, yet the H substance is found in almost all red cells and the O is comparatively rare (Morgan and Watkins, 1948). The ease with which the specificity of all types of cells is degraded so that they become agglutinable by all types of sera, suggests that the basic structure of all group substances is the same and that the specificity is due to an additional molecule (or molecules) in the nature of a hapten; and that it is this hapten which is destroyed by the enzyme processes responsible for panagglutination and haemagglutination.

CONCLUSIONS

The enzymes destroying blood group substance A and the R.D.E. were found to be produced by strains of *Cl. welchii* belonging to the four types A, B, C and D, and there appeared to be a strong correlation between the production of each of these enzymes and the haemagglutinin. Moreover the three activities could be inhibited by the same antisera to approximately the same titre. Other findings were that the HA enzyme produced panagglutination of R.B.C.—a phenomenon known to be produced by the R.D.E; and that blood group substance A caused inhibition of the HA. The suggestion is made, therefore, that the three activities, haemagglutination, destruction of virus receptors and destruction of blood group substance A, are due to the same enzyme.

ACKNOWLEDGMENTS

The work reported here formed part of a thesis for the degree of M.V.Sc. at the University of Liverpool.

I am grateful to Professor Glover for his interest and advice throughout the work. My thanks are also due to Dr. C. H. Andrewes, of the World Influenza Centre, who supplied the influenza virus; to Dr. A. W. Stableforth, of the Ministry of Agriculture, Fisheries and Food, Veterinary Laboratory, Weybridge, for the Newcastle Disease Virus; and to the Regional Blood Transfusion Service, Liverpool for the Group A red cells and the anti-A and anti-B sera.

REFERENCES

Beveridge, W. I. B., and Burnet, F. M. (1946). *Spec. Rep. M.R.C. Ser. No.* 256. H.M. Sta Off; London.
Boyd, W. C., and Shapleigh, E. (1954). *J. Immunol.*, **73**, 226.
Burnet, F. M., McCrea, J. F., Anderson, S. G. (1947). *Nature*, **160**, 404.
Burnet, F. M., McCrea, J. F. Stone, J. (1946). *Brit. J. exp. Path.*, **27**, 228.
Chase, M. W. (1938). *J. Bact.*, **36**, 383.
Dafaalla, E. N., and Soltys, M. A. (1953). *Nature*, **172**, 38.
De Burgh, P., Pen-Chung, Y., Howe, C., and Boranick, M. (1948). *J. exp. Med.*, **87**, 1.
Freidenreich, V. (1928). *Acta. Path. Microbiol. scand.*, **5**, 59.
Freidewald, W. F., Muller, E. S., Whatley, L. R. (1947). *J. exp. Med.*, **86**, 65.
Green, R. H., and Wooley, D. W. (1947). *Ibid.*, **86**, 55.
Gürturk, S. (1952). *Z. Hyg.*, **133**, 573.
Hirst, G. K. (1948) *J. exp. Med.*, **87**, 301.
Katitch, R. V. (1954). *Rev. d'Immunologie*, **18**, 199.
Landsteiner, K., and Chase, M. W. (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 713, 1208.
McCrea, J. F. (1947). *Aust. J. exp. Biol.*, **25**, 127.
Morgan, W. T. J. (1946). *Nature*, **158**, 759; (1947). *Experientia*, **3**, 257.
Morgan, W. T. J., and Watkins, W. M. (1948). *Brit. J. exp. Path.*, **29**, 159.
Oakley, C. L., and Warrack, H. G. (1953). *J. Hyg.*, **51**, 102.
Salk, J. E. (1944). *J. Immunol.*, **49**, 87.
Schiff, F. (1939). *J. infect. Dis.*, **65**, 127.
Stack, M. V., and Morgan, W. T. J. (1949). *Brit. J. exp. Path.*, **30**, 470.
Stone, J. D. (1947). *Aust. J. exp. Biol.*, **25**, 137.
Thomsen, O. (1926). *Z. Immun. Forsch.* **52**, 85.
Wickham, Nancy (1956). *J. Comp. Path.;* **66**. 62.

[*Received for publication, August 13th, 1955.*]

GIGANTOCOTYLE EXPLANATUM CREPLIN (TREMATODA: PARAMPHISTOMIDAE) INFECTION OF THE LIVER OF THE BUFFALO IN CEYLON

By

C. Kulasiri* and R. D. Seneviratne

Medical Research Institute, Colombo, Ceylon.

INTRODUCTION

Crusz (1952) carried out an important and extensive survey into the nature, incidence and geographical distribution of amphistomiasis in Ceylon.

Gigantocotyle explantum has been recorded in the liver of the buffalo in Ceylon, India, Burma, French Indo-China, the Philippines and the Celebes Islands. It has also been recorded in the liver of the zebu, *Bos taurus indicus* Linnaeus in Ceylon, India, Burma and the Philippines (Näsmark, 1937; Willmott, 1950; Crusz, 1952; Yamaguti, 1954). The incidence in Ceylon in the buffalo is high whereas in the zebu it is low (Crusz, 1952).

As no systematic study of the pathology of such infections has been attempted, the present work was undertaken to evaluate the nature of the damage caused by the economically important amphistome, *Gigantocotyle explanatum* in the liver of the buffalo, *Bubalus bubalis* Linnaeus.

MATERIALS AND METHODS

The infected livers, collected from buffaloes killed at the Colombo abattoir, were washed to remove the surface blood and brought to the laboratory. The surface was examined for gross pathological changes and affected areas were fixed in formol-saline. The bulk of the material was sliced to expose a large surface and immersed in a bath of 15 to 20 per cent formaldehyde. Representative groups of flukes from each specimen were liberated and fixed in formol-saline for systematic studies.

Serial sections 10μ thick were made of the formol-saline, as well as the formalin, fixed material and stained in Delafield's haematoxylin and eosin for routine examinations. Other sections were stained with van Gieson's stain and Weigert's haematoxylin. Whole flukes fixed in formalin or formol-saline were stained in acetic acid alum-carmine.

RESULTS

The Parasite

A description of the adult parasite is not included here as it may readily be found in many papers. However, the pre-adult stage (Fig. 1) encountered inside a thrombosed vessel needs description. The plane of sectioning is obliquely longitudinal but the parasite seems to be twisted between the two suckers. The total number of

*Present address : London School of Hygiene and Tropical Medicine, London.

sections in which the parasite appears is sixteen, but it is possible that some of the sections may be missing. The length of the fluke is 490μ, the breadth above the posterior sucker is 160μ and the depth (measured at the same level as the breadth) 159μ. The posterior sucker is terminal while the anterior sucker is sub-terminal. No sections passed through both suckers. The anterior sucker is pear-shaped and is 158μ long. The greatest width is 111μ while the depth is 100μ. The posterior sucker is 175μ long, 222μ broad and 80μ deep. From these measurements it is evident that the fluke is conical. As this description is based on a single specimen which may have been distorted either through being in tissue or due to fixation, these measurements may not be fully representative but they are in close agreement with those obtained from a similar fully calcified specimen encountered blocking a vein. The alimentary canal is wavy and extends up to the posterior sucker. It is lined in the mid-region by cells with darkly staining nuclei. No reproductive organs are present.

Since the suckers are at either end of the parasite it falls into the family Paramphistomidae (Trematoda). The posterior sucker is considerably larger than the anterior sucker and the ratio of the posterior sucker to the length of the body falls within the figures given by Näsmark (1937). On these characters the parasite is identified as a pre-adult of *G. explanatum.*

Pathology of the Infected Livers

The capsule is thickened and opaque and an occasional nodule or fibrous tag projects from the surface. The general contours are not visibly altered but the liver substance feels tougher than normal. On section numerous bile ducts are seen as cystic spaces or cylindrical dilatations measuring 1 mm. to 5 mm. in diameter packed with sexually mature adult parasites (Fig. 2). The invasion of the bile ducts appears uniform in all parts of the liver with no evidence of a preference for any particular lobe or region. A very striking phenomenon, however, is that the gall bladder although thickened and fibrosed never contains parasites. The dilated cystic duct is generally packed with parasites right up to the neck of the gall bladder (Fig. 3).

Histopathology

The capsular thickening is composed of a laminated collagenous membrane and fibroblastic layer. Immediately underneath, focal collections of cells or strands of fibroblasts stretch deep into the liver substance (Fig. 4). Dilatations of hepatic and portal veins and central sinusoids are a prominent feature.

Occasional foci of necrosis or haemorrhage involving a few parenchymatous cells or larger areas sometimes involving one or more lobules are present (Fig. 5). In nearly every section there are local collections of small round cells. The portal tract appears to be

most severely affected. The earliest lesion is a cellular infiltration of histiocytes, fibroblasts and other mononuclear cells with an occasional neutrophil and eosinophil. Fibroblasts later increase in numbers and collagenous fibres are laid down particularly round the bile ducts and blood vessels. At a later stage the portal tract appears as an irregular island of young fibrous tissue containing the usual triad of portal vein, hepatic artery and bile duct, each of which begins to show changes (Fig. 6). The vein is dilated and its scanty muscular layer is irregular and widened by fibrous tissue. Perivascular fibrosis is a conspicuous feature of the picture. Occasionally an organizing thrombus partially occludes the lumen. The hepatic artery shows medial hypertrophy which eventually appears as a massive ring of muscle fibres many times the normal thickness. Some of the fibres later atrophy and are replaced by patchy fibrosis. Sub-endothelial connective tissue proliferation goes on at the same time and reduces the lumen to a narrow cleft. Peri-vascular fibrosis is also prominent around the artery. Bile ducts in the portal tracts are generally dilated and some muscular hypertrophy and peri-ductal fibrosis are not uncommon. Fibrous bands from portal tracts spread out into the liver parenchyma and isolate islands of liver cells in their immediate vicinity. The lesions described above are seen in nearly every portal tract, the picture being that of a monolobular cirrhosis.

The parasites occur in three different sites, the liver substance, portal veins and bile ducts. One adult was found in a pool of blood in the liver substance. There was no inflammatory reaction and hence migration after death of the host cannot be excluded in this particular instance. On more than one occasion dead parasites were seen in the liver substance undergoing calcification and surrounded by a dense round cell infiltration. A pre-adult parasite was encountered in a vein embedded in an organizing thrombus. No degenerative changes were seen and hence it was presumed to be alive at the time of fixation. The vein was dilated and the walls fibrotic. In another vein the calcified remains of a parasite occupied the whole lumen. The wall of the vein was dilated and fibrosed.

Most of the parasites are found in dilated bile ducts (Fig. 2), the outermost layers of which show irregular discontinuous strands of atrophied smooth muscle separated by fibrous tissue and infiltrated with round cells which also infiltrate the adjacent liver paranchyma. Numerous small arterioles are seen with greatly hypertrophied media and their lumina almost obstructed by endarteritis.

The cellular infiltration is continued into the sub-mucosa and the mucosa of the bile ducts. The mucosa is hypertrophied and its deeper layers contain many mucous secreting glands. More superficially there are glandular spaces lined by tall columnar epithelium proliferating into papillomatous projections. The surface is necrotic in places and often covered with inflammatory exudate or mucus. Squamous metaplasia is occasionally seen. Within the lumen the adult parasite is found with its posterior sucker closely applied to the

mucous membrane. A flask-shaped projection of the mucosa is sucked in, filling the concavity of the large posterior sucker (Fig. 2).

The gall bladder mucosa is atrophied and its glandular epithelium is for the most part absent. A few scattered groups of mucous glands are seen in a scanty cellular connective tissue stroma containing dilated blood vessels. The muscle layer appears hypertrophied but is stretched and broken up by patchy fibrosis. The overall picture is that of muscular hypertrophy followed by pressure atrophy.

DISCUSSION

Näsmark (1937) differentiates *Gigantocotyle explanatum* from *G. bathycotyle* (Fischoeder) on the relative position of the testes i.e. in *G. explanatum* the testes lie diagonally and in *G. bathycotyle* they lie one behind the other. The presence of both types with transitional forms in the material examined here suggest that the two species are identical. Crusz (1952) and Nasmark (1937) himself are doubtful of the distinctions which have been drawn between the two species. The morphology of the species described by Willmott (1950) as *G. bathycotyle* from the rumen of the zebu in Ceylon and by Yamaguti (1954) as *Explanatum explanatum* Creplin from the buffalo in the Celebes Islands is similar to the material examined here. These two species are therefore referred to as *G. explanatum* Creplin.

The pathological picture of the material studied suggests a transperitoneal infection of the bile ducts such as occurs in *Fasciola hepatica*. (Shaw ,1932; Schumacher, 1938; Morrill and Shaw 1942; Krull and Jackson, 1943). In the many sections examined, only on two occasions were flukes encountered within veins. One of them was enclosed in granulation tissue and the other was calcified, indicating that they do not commonly travel in veins or survive in them for long.

The monolobular type of cirrhosis is the expected result of widespread blocking of bile ducts by the parasite. The histological changes caused by the parasite within the bile ducts call for no comment but the extraordinary muscular hypertrophy and sub-endothelial proliferation of vessels particularly the hepatic arteries need an explanation. It is suggested that this type of reaction is the result of a hepatic hypertension resulting either from a diffusible chemical substance or substances elaborated by the parasite or from widespread granulomatous lesions in the portal tracts involving blood vessels.

Willmott (1950) reports that her specimens came from the rumen of the zebu in Ceylon. Crusz (1952) in his extensive survey found no specimens of *Gigantocotyle* in any site other than the liver and does not mention whether they were present in the gall bladder. Näsmark (1937) points out that *G. explanatum* has not been found elsewhere than in the liver, bile ducts and gall bladder, except in one collection which came from the duodenum. He does not comment on the site of infection of *G. bathycotyle* although in some of the collections which he examined the stomach had been cited. He mentions however

that if any location outside the liver is possible it would be the duodenum and not the stomach. Although he was aware of Willmott's work, Crusz (1952) does not comment on her finding the parasite in the rumen and further work is required to verify whether it does occur in this abnormal site.

In the livers examined here, the parasite was not encountered in the gall bladder. Further, the pathological picture does not suggest an infection at this site, although in some heavily infected livers the parasites reached up to the neck of the gall bladder. Three reasons may be suggested to explain this observation. Firstly the structure of the wall of the gall bladder and its position between the lobes of the liver may prevent the migrating young flukes reaching it from the peritoneal cavity. Secondly the infection via the cystic duct may be obstructed by the sphincter muscle at the mouth of the gall bladder. Finally the concentration of the bile in the gall bladder, being higher than that normally encountered in the bile duct (Kleiner, 1951), may have an adverse effect on the migration of the flukes to this site. This physiological reason may supplement to a great extent the mechanical obstruction caused by the previous two.

CONCLUSIONS

Gigantocotyle explanatum Creplin, of which *G. bathycotyle* and *Explanatum explanatum* are considered to be synonyms, was the only amphistome found in the liver of the buffalo in Ceylon. It was encountered in three different sites: liver substance, portal veins and bile ducts. The pre-adult stage in the portal vein is described.

The histopathology of the liver has been studied and tentative explanations of the changes observed are suggested. The evidence points to a transperitoneal path of infection. The absence of the parasite from the gall bladder is noted and possible reasons for this are discussed.

ACKNOWLEDGMENTS

We are extremely grateful to Dr. D. W. Amerasingha, Chief Veterinary Surgeon, Colombo Municipal Council and Dr. S. Saravanamuttu, Superintendant, Slaughter House, Colombo for the material used in this study; to Mr. Y. Wijayaratnam for technical assistance and to Mr. R. Surendranathan for the photographs.

REFERENCES

Cruz, H. (1952). *Ceylon J. Sci.* (B) **25**, 59.
Kleiner, I. S. (1951). *Human Biochemistry.* London: Henry Kimpton.
Krull, W. L., and Jackson, R. S. (1943). *J. Wash. Acad. Sci.*, **33**, 79.
Morrill, D. R., and Shaw, J. N. (1942). *Bull. Oreg. agric. Exp. Sta.*, No. 48.
Näsmark, K. (1937). *Zool. Bidr.* Uppsala., **16**, 301.
Schumaker, W. (1938). *Z. Parasitenk.*, **10**, 608.
Shaw, J. N. (1932). *J. Amer. vet. med. Ass.* **81**, 76.
Willmott, S. (1950). *J. Helminth.*, **24**, 1.
Yamaguti, S. (1954). *Acta Medicinae Okayama.*, **8**, 541.

[*Received for publication, June 20th,* 1955]

LEGENDS TO ILLUSTRATIONS

Fig. 1. Longitudinal section of a pre-adult parasite through the posterior sucker showing alimentary canal lined by darkly staining cells. H. & E. X 80.

Fig. 2. Transverse section of a dilated bile duct containing several adult parasites. At the lower left corner a flask-shaped projection of the mucosa is seen within a posterior sucker. H. & E. X 20.

Fig. 3. Dilated cystic duct is seen on the left packed with adult parasites. On the right the thickened and fibrosed gall bladder is seen completely free of parasites. H. & E. X 1.1.

Fig. 4. A fibroblastic thickening of the capsule is seen above. A collection of fibroblasts and small round cells is seen immediately underneath stretching into the liver substance. Dilated portal and hepatic veins and sinusoids are very evident. H. & E. X 20.

Fig. 5. A necrotic focus infiltrated with inflammatory cells. H. & E. X 80.

Fig. 6. A fibrotic portal tract showing a dilated bile duct on the left, a vein on the right and several arteries with medial hypertrophy and sub-endothelial proliferation. H. & E. X 20.

FIG. 1

FIG. 2

To face page 88

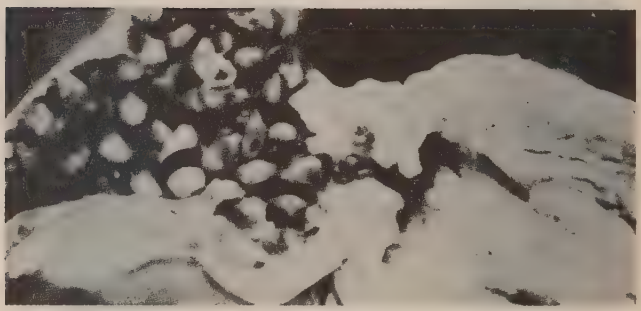


FIG. 3

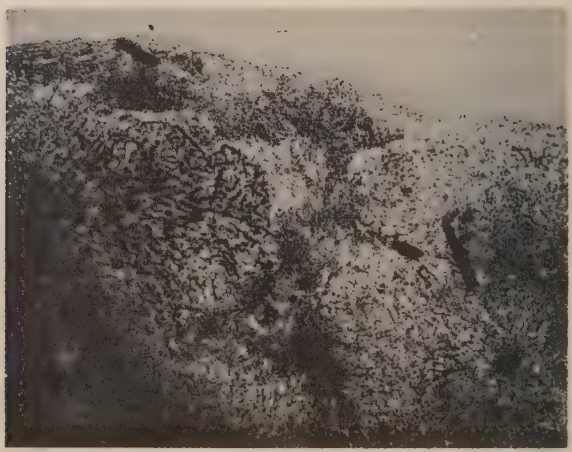


FIG. 4

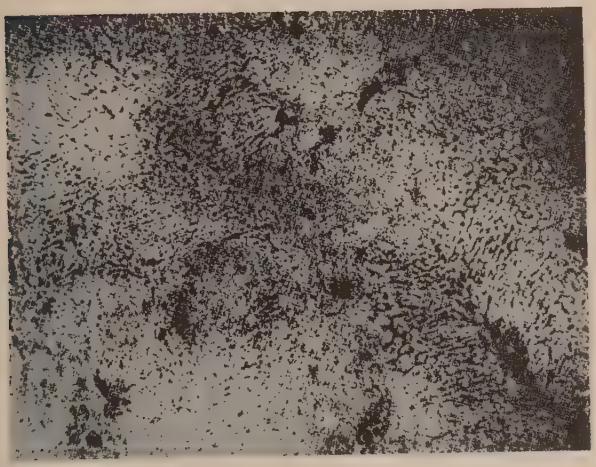


FIG. 5

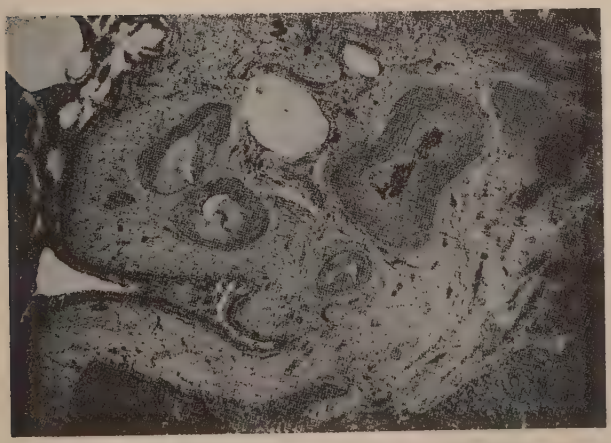


FIG. 6

THE ACTION OF ANTIBIOTICS AND SULPHAMEZATHINE ON THE CAUSAL AGENT OF VIRUS PNEUMONIA OF PIGS

By

A. O. Betts

Department of Animal Pathology, University of Cambridge

and

R. C. Campbell

Agricultural Research Council Statistics Group, School of Agriculture, University of Cambridge.

INTRODUCTION

Although a few reports have been made concerning the clinical improvement produced by antibiotics in pigs suffering from virus pneumonia of pigs (V.P.P.) (Penny, 1954; Börnfors and Lannek, 1955), no experimental work on the action of antibiotics upon the causal agent of V.P.P. has been reported apart from preliminary notes (Betts, 1952; Beveridge and Betts, 1953).

The purpose of the experiments described in this paper was two-fold, firstly to try to find an agent which might be of use in the treatment or prevention of virus pneumonia, and secondly to characterize further the causal agent. Virus pneumonia resembles in several respects grey lung disease of mice and primary atypical pneumonia of man, both of which conditions are susceptible to treatment with chlortetracycline (aureomycin) and oxytetracycline (terramycin) (Andrewes and Niven, 1950; Meikeljohn and Shragg, 1949). Hence the tetracycline group of antibiotics was tested as well as penicillin, streptomycin, chloromycetin and sulphamezathine.

MATERIALS AND METHODS

Experimental animals. Except where otherwise stated the pigs were between eight and sixteen weeks of age at the start of each experiment and came from the herd at the Department of Animal Pathology, which is free from virus pneumonia.

Method of infection. The M.R. strain of virus (Betts and Beveridge, 1953) was used for experimental infection. The method was that described by those authors and the dilution of inoculum was 10^{-4} except where otherwise stated. All inocula were free from bacteria as judged by routine culture of the suspensions on blood-agar plates under aerobic conditions.

Recording of results. The incidence of coughing and of other clinical symptoms in the living animals was noted and the extent of lesions at slaughter was recorded. In some instances recovery of the virus was attempted by inoculation into other pigs.

Biometrical methods. The allocation of pigs to groups was randomised, the groups being balanced as far as possible for litter, sex and

weight. The results (the numbers infected and non-infected in the various treatment groups) were analysed as contingency tables, the differences between the treatments being measured by χ^2.

TABLE 1

SUMMARY OF THE TYPES OF ACTION STUDIED AND OF THE RESULTS OBTAINED

Agent	*Action in vitro*	*Prophylactic action*	*Curative action*
Sulphamezathine (I.C.I.)	Not tested	No effect	Not tested
Penicillin	No effect	No effect	(Tested together)
Streptomycin	No effect	Slight effect	No effect
Chloramphenicol (Chloromycetin, Parke Davis)	Not tested	No effect	No effect
Chlortetracycline (Aureomycin, Lederle) (*a*) pure	No effect	Marked effect	No effect
(*b*) feed supplement	Not tested	No effect	No effect
Oxytetracycline (Terramycin, Pfizer)	Not tested	Marked effect	No effect
Tetracycline (Achromycin, Lederle)	Not tested	Not tested	No effect

RESULTS

The in vitro, prophylactic and curative actions of the various agents, which were not all tested for each agent, are discussed separately in this section. The tests carried out and the results obtained, are summarized in Table 1.

Action in vitro

Penicillin, streptomycin and chlortetracycline were studied, but had no effects on the virus.

Penicillin. A suspension of pneumonic lung tissue was prepared in phosphate buffer to give a final dilution of 10^{-2} and was lightly centrifuged. Penicillin was added to the supernatant fluid to give a concentration of 500 i.u. per ml. The inoculum was left at room temperature and portions inoculated into pigs 0, 4, 6 and 24 hours after preparation. The results of this experiment are shown in Table 2. Subsequent to this experiment, many inoculations have been made using unfiltered lung suspensions to which penicillin and streptomycin have been added to suppress bacteria. Even in as high concentrations as 2,000 units of each antibiotic per ml. there has been no deleterious action on the virus.

TABLE 2

THE EFFECT OF PENICILLIN IN VITRO

Time at room temperature	*Number of pigs inoculated*	*Days between inoculation and slaughter*	*Lesions at slaughter*
0 hrs.	1	11	+
4 hrs.	1	11	+
6 hrs.	1	18	+
24 hrs.	3	11, 20, 20	+,+,+

+ = lesions of pneumonia.

Streptomycin. Although no specific trials have been made to test the effect of streptomycin in vitro, on numerous occasions streptomycin concentrations up to 2000 'S' units per ml. have been added to unfiltered lung suspensions in order to suppress bacteria and have had no obvious detrimental effect on the virus.

Chlortetracycline. (Aureomycin). A 10^{-2} dilution of infected lung tissue was prepared in phosphate buffer at pH 7.2 and the suspension was divided into two portions. Aureomycin was added to each portion to give a concentration of 100μg. per ml., it having been determined previously that this concentration did not affect the pH of the suspension. The two bottles were wrapped in black paper and left at room temperature. After 6 hours, 20 ml. amounts from one portion were inoculated into each of two pigs. The two pigs began to cough within two weeks and when they were killed 22 days after inoculation both were found to have extensive lesions of virus pneumonia.

Twenty-four hours after preparation, the remaining portion of the suspension was inoculated into two other pigs. Both pigs were coughing before they were killed 21 days after inoculation, at which time both were found to have extensive pneumonic lesions.

Prophylactic Action

The prophylactic actions of all the agents except tetracycline were studied; chlortetracycline was also tested as a feed supplement. Streptomycin had a slight action while chlortetracycline (pure) and oxytetracycline gave very considerable protection, but none of the other substances had any effect.

Sulphamezathine (*I.C.I.*)

Two experiments were carried out to determine whether this drug could prevent infection. The drug was administered in the form of sulphamezathine sodium 33$\frac{1}{3}$ per cent solution at the rate of 0.1 g. of solid drug per kg. bodyweight for the first day, and at half this rate subsequently. The daily dose was given in one injection, subcutaneously behind the ear. In one experiment, dosing was continued for 3 days and in the other for 7 days. About two hours after the first

injection of sulphamezathine the treated pigs and the controls were infected with virus. All the pigs were slaughtered 21 days after inoculation. The results of these experiments are given in Table 3.

All the pigs in both groups were coughing regularly before slaughter, and in the second experiment one of the treated pigs began to cough even before the final injection of sulphamezathine. There was no measurable difference between the extent of lesions in the control pigs and in the treated pigs.

TABLE 3

THE PROPHYLACTIC EFFECT OF SULPHAMEZATHINE

Experimental group	*Number of pigs*	*Length of treatment*	*Days between inoculation and slaughter*	*Lesions at slaughter*
Exp. 1. Treated	2	3 days	21	+,+
Exp. 1. Control	2	—	21	+,+
Exp. 2. Treated	3	7 days	21	+,+,+
Exp. 2. Control	2	—	21	+,+

+ = lesions of pneumonia.

Penicillin

In two experiments designed to test whether penicillin could prevent infection becoming established, pigs were injected in the muscles of the buttocks with procaine penicillin suspension in the form of "Distaquaine 'G'" (May & Baker) at the rate of 4,000 i.u. per lb. body-weight per day. The penicillin was given once daily and dosing was continued for 3 days in one experiment and for 7 days in the other. Five hours after the first "Distaquaine" injection the treated and control pigs were inoculated with the virus. The results are shown in Table 4. The lesions in the penicillin-treated animals were more extensive than those in the controls.

TABLE 4

THE ACTION OF PENICILLIN IN VIVO

Experimental group	*Number of pigs*	*Length of treatment*	*Days between inoculation and slaughter*	*Lesions at slaughter*
Group A. Treated	2	3 days	26	+,+
Group A. Control	2	—	26	+,+
Group B. Treated	4	7 days	21	+,+,+,+
Group B. Control	5	—	21	+,+,+,+,?

+ = positive lesions. ? = doubtful lesion.

Streptomycin

Two experiments were undertaken to test whether streptomycin could prevent the establishment of infection. Dosage was at the rate of 20 mg. of streptomycin per lb. body-weight per day. The antibiotic was given twice daily at 12-hour intervals by subcutaneous injection and treatment was continued for 7 days in each case. The first injection of streptomycin was given five hours before inoculation of the virus. The results are given in Table 5. Some slight protection appeared to be given by the antibiotic, but where lesions were present in the treated pigs they were as extensive as those in the control animals.

TABLE 5

THE EFFECT OF STREPTOMYCIN IN VIVO

Experimental group	*Number of pigs*	*Length of treatment*	*Days between inoculation and slaughter*	*Lesions at slaughter*
Group 1. Treated	4	7 days	22 days	+,+, 0, 0
Group 1. Control	5	—	22 days	+,+,+,+, +
Group 2. Treated	12	7 days	24 days	+,+,+,+,+,+ +,+,+, 0, 0, 0
Group 2. Control	12	—	24 days	+,+,+,+,+,+ +,+,+,+,+,+

+ = lesions present. 0 = no lesions present.

Chloramphenicol (Chloromycetin)

Twenty newly weaned pigs were divided into two groups of ten. One group was injected by intramuscular injection in the buttocks with chloramphenicol at the rate of 5 mg. per lb. body-weight per day. The daily dose was divided into three and one third given at 8-hour intervals. Dosing was continued for seven days. Three hours after the first injection all the treated and control pigs were inoculated with virus. The onset of coughing was slower in the treated pigs but all the pigs were coughing by the time they were killed on the 21st day following infection. At slaughter there was no measurable difference between the extent of lesions in the lungs of pigs from the treated and control groups or in the degree of enlargement of the associated lymph nodes.

Chlortetracycline (Aureomycin)

The pure antibiotic. When the first experiments with chlortetracycline were undertaken in 1951, techniques for the regular infection of pigs had not been perfected: moreover, the antibiotic was very scarce and was only available in the form of capsules. In this form it proved somewhat irritant to the pig's gastric mucosa with the result that a few pigs vomited after dosing: consequently the amount of antibiotic actually absorbed was irregular. In all, 8 pigs were dosed

by this route at the rate of 25 mg. of chlortetracycline per kg. body-weight per day. The daily dose was divided into three portions. One portion was given every eight hours and dosing was continued for 3 days. About 3 hours after the first dose of chlortetracycline had been given, the pigs were inoculated with suspensions of pneumonic lung tissue, and at the same time, 17 controls were also inoculated. The pigs were killed 21 days after inoculation. Of the 8 pigs treated, 5 had no lesions, 2 showed small areas of pneumonia and the lungs of the remaining pig were filled with inspired blood so that it was impossible to ascertain whether or not there were small areas of pneumonia. Fifteen of the controls had pneumonic lungs but the lungs of the other two were free from lesions.

Following these uncertain results a more drastic experiment was undertaken. The contents of the capsules were emptied out and immediately before dosing a suspension of the chlortetracycline powder was made in normal saline to give a concentration of about 25 mg. per ml. Three pigs were injected intraperitoneally with the freshly-made suspension at the rate of 25 mg. of chlortetracycline per kg. body-weight per day. One third of the daily dose was given every eight hours for six days. The irritant nature of the preparation produced considerable reaction at the site of the injection and there was some vomiting, presumably a reflex from peritonitis. Shortly after the first dose had been given, the 3 pigs and 6 controls were each inoculated with 20 ml. of a 10^{-5} infective lung suspension. When the pigs were killed 21 days after inoculation the lungs of the three treated pigs were completely free from lesions while all the controls had pneumonia. As expected the chlortetracycline had produced chronic peritonitis in the treated pigs.

Although these results were significant at the 0.1 per cent level, when an injectable form of chlortetracycline became available it was decided to repeat the experiment. Nine newly weaned pigs were divided into groups of four (treated) and five (controls). The pigs in the treated group were injected intravenously in the ear veins with "Aureomycin intravenous" at the rate of 5 mg. of antibiotic per lb. body-weight, one third of the daily dose being given at 8-hour intervals. Dosing was continued for 7 days. Three hours after the initial dose of antibiotic each pig in both groups was inoculated with virus. The first pig in the control group began to cough on the 11th day and all were coughing by the 18th day; there was no coughing in the treated group. When the pigs were killed on the 21st day, there were extensive lesions in the lungs of the five control pigs but the lungs of the treated pigs were free from lesions. Attempts to recover virus by pig-passage from the lungs of the treated pigs were unsuccessful but the virus was readily recovered from the lungs of the control pigs.

The results of these experiments are summarized in Table 6. The comparison of the proportions infected in the control and treated groups gave a highly significant χ^2 of 22.513 on 1 degree of freedom.

TABLE 6

THE PROPHYLACTIC EFFECT OF PURE CHLORTETRACYCLINE

Experiment	*Number of pigs*	*Dosage rate per day*	*Route of administration*	*Length of treatment*	*Days between inoculation and slaughter*	*Lesions at slaughter*	*Recovery of virus from lesions*
Exp. A. Treated	8	25 mg/Kg	Oral	3 days	21	+,+,0,0 0,0,0,?	Not attempted
Exp. A. Control	17	—	—	—	21	+,+,+,+ +,+,+,+ +,+,+,+ +,+,+,0 0	
Exp. B. Treated	3	25 mg/Kg	Intraperitoneal	6 days	21	0,0,0	Not attempted
Exp. B. Control	6	—	—	—	21	+,+,+,+ +,+	
Exp. C. Treated	4	5 mg/lb	Intravenous	7 days	21	0,0,0,0	Negative
Exp. C. Control	5	—	—	—	21	+,+,+,+ +	Positive

+ = lesions of pneumonia. 0 = no lesions present. ? = doubtful.

Impure feeding supplements. Four pigs were fed Aurofac 2A, which contained 3.6 g. of aureomycin per lb., at a rate equivalent to 30 mg. of aureomycin per kg. body-weight per day. This was mixed with the food and was fed twice daily for 7 days. The pigs apparently found the mixture to be quite palatable and no digestive disturbances resulted. A few hours after the first feed on the experimental ration the four treated pigs and five control pigs were inoculated with virus. The Aurofac-fed pigs showed no symptoms before they were killed 21 days later, whereas all the control pigs were coughing by that time when they were also slaughtered. Nevertheless, the lungs of all the pigs were pneumonic, although the lesions in the treated pigs were paler, almost translucent and much more grey than those in the controls.

Oxytetracycline (Terramycin)

At the time these experiments were begun oxytetracycline was available only in the pure crystalline form for intravenous injection, but subsequently it became available in a form suitable for intramuscular injection. By the use of these pure injectable forms, uncertainty concerning the dosage of the antibiotic was eliminated.

In the first experiment five pigs were given oxytetracycline in saline at a contration of 15 to 20 mg. per ml. and at a dosage rate of 5 mg. per lb. body-weight. The daily dose was divided into two equal amounts which were injected intravenously at 12-hour intervals and treatment was continued for four days. Three hours after the initial injection of oxytetracycline these five pigs and four controls were inoculated with virus. Seven days after inoculation all four of the control pigs were coughing and thereafter they coughed continually, whereas the treated pigs developed no symptoms. In view of the marked symptoms in the controls and having regard to previous experience, it was not considered necessary to kill more than one of the controls at the same time as the treated pigs. (The other three were retained for another experiment necessitating the use of infected pigs). The group of five treated pigs and the one control were killed on the 18th day after inoculation, when the latter pig was found to have extensive pneumonia with marked enlargement of the thoracic lymph nodes, while the lungs of the pigs which had received oxytetracycline were completely free from lesions and the associated lymph nodes were normal.

In a second similar experiment, five pigs were treated with the intramuscular form of the antibiotic. Dosage was at 5 mg. per lb. body-weight per day, one third of the dose being given at 8-hour intervals and dosage continued for 7 days. Coughing in the five controls began on the 8th day but there was no coughing in the treated pigs. When all the pigs were slaughtered on the 21st day the controls had extensive pneumonic lesions, whereas the lungs of the treated pigs were free from lesions, although minute areas of collapse were present in both cardiac lobes of one pig. It was not

possible to recover virus from the treated pigs by inoculation of material into other pigs, but virus was readily recovered from the control pigs. The prophylactic results with oxytetracycline are summarized in Table 7; the comparison of the proportions infected in the control and treated groups gave a highly significant χ^2 of 11.992 on 1 degree of freedom.

Curative Action

The curative actions of all the agents except sulphamezathine were studied, penicillin and streptomycin being tested together, and chlortetracycline being used both as a feed supplement and as the pure antibiotic. No curative action was found.

Penicillin and streptomycin, chloramphenicol, chlortetracycline (pure)

When thirteen naturally infected pigs became available for experiment, the opportunity was taken to test the curative effect of some of the antibiotics. Four pigs were treated with penicillin and streptomycin together, four with chloramphenicol, four with chlortetracycline and one control left untreated. The respective daily doses of the antibiotics were 1,000 i.u. per lb. body-weight (penicillin), 5,000 'S' units per lb. body-weight (streptomycin), 50 mg. per kg. body-weight (chloramphenicol) and 25 mg. per kg. body-weight (chlortetracycline). The penicillin and streptomycin were given by intramuscular injection into the buttocks, but the chloramphenicol and chlortetracycline were available only in capsules and so were given by mouth. In all cases the daily dose was divided into three portions, and one portion given every 8 hours; dosing was continued for four days. Two pigs from each treated group were killed on the 18th day, and the remaining animals, including the control, on the 32nd day. Coughing was markedly reduced in all the treated pigs which also improved in condition, but at slaughter no differences could be found between the lungs of the treated and control animals, either in extent of lesions or in degree of resolution.

In a second experiment using only chlortetracycline in the treatment of clinical cases, the pure crystalline intravenous form was available. There were five pigs in the treated group and four untreated controls. The antibiotic was administered twice daily at 12-hour intervals by intravenous injection into the ear veins. Treatment was continued for 7 days. The dosage rate used was 7 mg. per lb. body-weight per day. Coughing disappeared in the treated group, but when all the pigs were killed 30 days after the beginning of the trial, pneumonia was still present to the same extent as in the controls.

Penicillin and chlortetracycline (as impure feeding supplements)

An experiment to study the effect of feeding high concentrations of Aurofac 2A to infected pigs was done at the National Institute for Research in Dairying, Shinfield, in collaboration with Dr. Kon and

TABLE 7

THE PROPHYLACTIC EFFECT OF OXYTETRACYCLINE

(5 mg. per lb. body-weight)

Experimental group	*Number of pigs*	*Length of treatment*	*Days between inoculation and slaughter*	*Lesions at slaughter*	*Recovery of virus*
Group 1. Treated	5	4 days	18 days	0, 0, 0, 0, 0	Not attempted
Group 1. Control	4	—	18 days	+, (remaining 3 positive clinically)	Not attempted
Group 2. Treated	5	7 days	2 days	c, 0, 0, 0, 0	Negative
Group 2. Control	5	—	21 days	+,+,+,+,+	Positive

+ = lesions present. 0 = no lesions. c = small areas of collapse.

Dr. Mitchell. Twenty-four naturally infected pigs were available and these were divided into four groups of six which were treated as follows: (*a*) Aurofac 2A fed at the level of 56.7 g. per 100 lb. of food; (*b*) Procaine penicillin fed at the level of 625 mg. per 100 lb. of food; (*c*) Aurofac 2A at 6 lb. per 100 lb. of food (approximately 18 mg. of pure aureomycin per kg. body-weight per day) for two weeks, then Aurofac as in (*a*) for four weeks, then the six-week cycle repeated; (*d*) Procaine penicillin at 34.6 g. per 100 lb. of food for two weeks, then as in (*b*) for four weeks, then the six-week cycle repeated.

Treatments (*b*) and (*d*) may be regarded as controls, since these levels of penicillin have been shown to have no effect on the virus.

The pigs in all the groups were weighed weekly and coughing was recorded daily. Coughing rapidly diminished in the group being fed the high level of Aurofac, and, before the end of the first fortnight, coughing had disappeared completely from the pigs of this group. These pigs had a sheen on their coats and looked better than those in the other groups. Coughing in the other groups also diminished but did not disappear completely. All the pigs were killed 12 weeks after the experiment began. There was no obvious difference in the extent of lesions, nor in the degree of resolution of lesions between pigs of the four groups.

Oxytetracycline

One experiment was undertaken to determine whether or not oxytetracycline was of value as a therapeutic agent in the treatment of virus pneumonia. For convenience of administration, the preparation for intramuscular injection was used, injected into the buttocks of five clinical cases, at the rate of 7 mg. per lb. body-weight per day. Four similar clinical cases were left untreated as controls. The solution of antibiotic was made in saline to give a concentration of less than 20 mg. per ml. At this dilution no reaction occurred at the site of injection in spite of the frequency of administration. The calculated daily dose was divided into two equal amounts and each half given at 12-hour intervals for seven consecutive days. As with aureomycin, the treated pigs ceased to cough, and improved in condition. When, however, the pigs were killed on the 30th day, again as in the case of those treated with aureomycin, the lungs were still pneumonic and little different in appearance from the controls.

Tetracycline (Achromycin)

One experiment was carried out to test whether the newer antibiotic tetracycline, which is derived from chlortetracycline, had any therapeutic action against the M.R. strain of virus. Twenty-four pigs were randomised into six groups of four and all were inoculated with virus. Coughing began on the 7th day and by the 15th day all the pigs were coughing. Twenty-four days after infection three of the groups were treated for 7 days with tetracycline by intramuscular injection at the rate of 5 mg. per lb. body-weight per day, the calcu-

lated dose being divided into three equal doses at 8-hour intervals. Coughing practically ceased in the pigs of the treated groups within a week, but re-appeared after a few weeks. Coughing in the controls lessened slowly. Pairs of groups were killed 29, 43 and 70 days from the commencement of treatment, and recovery of virus was attempted from the lungs of pigs in the first two pairs. The results of this experiment are shown in Table 8.

TABLE 8

THE THERAPEUTIC ACTION OF TETRACYCLINE

Group	*Days between beginning of treatment and slaughter*	*Presence of lesions*	*Recovery of virus*
A Controls	29	+,+,+,+	Positive
A Treated	29	+,+,+,+	Positive
B Controls	43	R,+,+,+	Positive
B Treated	43	+,+,+,+	Positive
C Controls	70	R,+,+,+	Not attempted
C Treated	70	R,R,+,+	Not attempted

+=Active lesions. R=Resolving lesions.

DISCUSSION

Evidence in the literature for the action of antibiotics on viruses, other than those of the psittacosis-lymphogranuloma group, is scanty. An exception to this is "grey-lung virus" of mice. Andrewes and Niven (1950) showed that this agent, like the causal agent of virus pneumonia of pigs, was susceptible to chlortetracycline and oxytetracycline but not to chloromycetin. In the case of "grey-lung virus", however, chlortetracycline and oxytetracycline not merely prevented lesions but also had a marked curative action, an effect we were unable to demonstrate with the pig agent.

Eaton (1950) reported that chlortetracycline, but not chloromycetin, was active in preventing infection with his "atypical pneumonia virus" in cotton rats, and that it also had a therapeutic action, an observation which is supported by various reports of the value of chlortetracycline in the treatment of primary atypical pneumonia in man (Meikeljohn and Shragg, 1949).

The action of antibiotics on the causal agents of "grey-lung disease", "atypical pneumonia of man" and "virus pneumonia of pigs" raises the question whether these agents are true viruses. In the case of the pig disease further doubts may be cast as a result of the slight action of streptomycin on the causal agent. On the other hand, like Andrewes and Niven (1950) in the case of the mouse agent, we have been unable to find any other evidence to suggest that the

causal agent of V.P.P. is not a virus. A colleague, Mr. P. Whittlestone, has isolated pleuropneumonia-like organisms from many cases of V.P.P. but it has not been possible to produce pneumonia with these organisms. Moreover, we have had no difficulty in reproducing the disease with material from which such pleuropneumonia-like organisms cannot be recovered.

If, then, the causal agent of V.P.P. is a virus, the results of the experiments described in this paper suggest that the action of the tetracycline group of antibiotics on the virus is likely to be at the stages of attachment of the virus to the cell or its penetration into the cell. These antibiotics appear to have little or no action on the agent once it is inside the cell and none when it is in the non-active state, as in a suspension of pneumonic tissue.

CONCLUSIONS

The effects of sulphamezathine, penicillin, streptomycin, chloromycetin, chlortetracycline, oxytetracycline and tetracycline on the causal agent of virus pneumonia of pigs were tested. It was found that infection could be prevented with chlortetracycline and oxytetracycline, but that neither of these antibiotics nor the other drugs tested had any effect upon established lesions. Streptomycin appeared to have some slight protective action.

ACKNOWLEDGMENTS

We are grateful to Messrs. P. T. Edwards and J. Gawthorp for their assistance and to Messrs. Parke-Davis & Co., Lederle & Co., and Pfizer & Co. who kindly supplied the chloramphenicol, chlortetracycline and tetracycline, and oxytetracycline respectively.

REFERENCES

Andrewes, C. H., and Niven, J. S. F. (1950). *Brit. J. exp. Path.*, **31**, 767.
Betts, A. O. (1952). *Vet. Rec.*, **64**, 283.
Betts, A. O., Beveridge, and W. I. B. (1953). *Ibid.* **65**, 515.
Beveridge, W. I. B., and Betts, A. O. (1953). *Proc. 15th Int. vet. Cong. Part II.*, 240.
Börnfors, S., and Lannek, N. (1955). *Nord. vet. Med.*, **7**, 15.
Eaton, M. D. (1950). *Proc. Soc. exp. Biol. N.Y.*, **73**, 24.
Meikeljohn, G., and Shragg, R. I. (1949). *J. Amer. med. Ass.*, **140**, 391.
Penny, R. H. C. (1954). *Vet. Rec.*, **66**, 730.

[*Received for publication, September 12th,* 1955].

THE EFFECT OF REDUCTION OF BACTERIAL INFILTRATION BY AUREOMYCIN ON THE NORMAL ALIMENTARY LYMPHOID TISSUE OF THE RABBIT

By

J. B. Enticknap*

Department of Pathology, Guy's Hospital Medical School

INTRODUCTION

The presence of bacteria in the lymphoid structures of the rabbit's appendix, as well as in the other parts of the gut, was first described by Bizzozero (1885) and has since been repeatedly confirmed. The literature has been reviewed by Digby and Enticknap (1954). It has now been observed by us without exception in about 300 rabbits. If the appendicular lumen is separated from that of the caecum and made to drain to the outside, while still retaining its blood and nerve supplies, it soon becomes emptied of bacteria. After about 2 weeks the number present in the submucosa is also reduced and thenceforward the lymphoid elements undergo progressive atrophy. This sequence of changes, which has also been observed by Buirge, Dennis, Varco and Wangensteen (1940), was frequently seen in the earlier rabbits submitted by Digby to isola-appendicostomy (Digby, 1950; Digby and Enticknap, 1954) and kept for several months after the operation.

The following communication describes the effects on the lymphoid structures of reducing the bacterial flora of the alimentary tract in the intact animal by the administration of aureomycin. A result similar to that which follows surgical isolation of the appendix was produced. It seems more probable therefore that the loss of lymphoid elements is due to the absence of bacteria from the lumen rather than to any post-operative displacement that disturbs its blood supply or secretory activity. A similar result was seen in limited experiments with bacitracin.

Four separate experiments have given similar results. Definitive changes followed the oral administration of 10 mg. aureomycin daily for a month and these are described in detail. Subsequently the same dose was given for shorter periods to discover when it first became effective and there were some preliminary experiments which helped to decide which drug to use and in what dosage.

MATERIAL AND METHODS

Animals. A total of 33 test and 18 control rabbits of all ages were used. Following a preliminary test to establish a suitable dose the main experiment was performed on 2 litters of 9 from sister grey does served by the same buck and a subsequent litter from the same buck and one of these does, the other animals being albinos. The 3 litters contained roughly equal numbers of each sex and they were allotted randomly into experimental

*Present address: East Ham Memorial Hospital, London, E.7.

groups of equal sex composition. When they were six weeks old the animals were observed for 2 weeks to make sure they were gaining weight satisfactorily and the experiment then started. At its conclusion the animals were killed by exsanguination under nembutal anaesthesia.

Drug administration. Aureomycin hydrochloride was supplied in the drinking water each morning in an effective dose of about 10 mg/kg body weight. Twice this amount was offered to allow for deterioration in the drug after solution and variation in water requirements.

Bacteriological examinations. To estimate changes in the gut flora faecal pellets were lightly ground in sterile tissue grinders and cultures made of known dilutions of the suspensions. At necropsy quantitative cultures and standardised smears for assessment of the flora by direct observation were made of gut contents from the junction of caecum and appendix. The number of organisms present in the smears was assessed into grades 1, 2 or 3, the group from which the specimen came being unknown to the observer. A small block of appendicular wall was removed with sterile precautions and placed upon the platform of a freezing microtome; a sterile razor was used to cut sections from and parallel to the serosal aspect. Cultures of submucosal bacteria were made by incubating in Robertson's meat medium the second 100 micron thick horizontal section. The area from which the cultures were made was embedded in paraffin and vertical sections were cut to ensure that the lumen was unopened.

Observations on tissues. The liver, heart, spleen, stomach, thymus, mesenteric lymphoid mass or pancreas of Aselli (m.l.m.), appendix, sacculus rotundus, proximal caecal patch (p.c.p.) and the constant terminal ileal patch (t.i.p.) were dissected free from fat and large vessels at necropsy and were weighed after formalin fixation. Paraffin sections were stained with haematoxylin and eosin for assessing the atrophy of lymphoid elements and with Gram's method to determine the degree of reduction of the bacterial infiltration. Representative sections of sternal bone marrow were also examined to assess the number of non-alimentary lymphocytes.

Observations on blood. To estimate any deficiency of aneurin metabolism, blood pyruvate levels were measured in 2 treated and 2 control animals 30 minutes after giving 200 mg/kg glucose intravenously; blood samples were collected by cardiac puncture directly into trichloroacetic acid. The animals were under nembutal narcosis during the whole period. Haemoglobin estimations, total and differential white cell counts and mean red cell diameter measurements were made on all animals and natural agglutinins for the organisms recovered from the gut were sought for.

RESULTS

Weight

The weights of the various lymphoid masses underwent progressive reduction during the course of aureomycin administration. The mean weights of these structures in six untreated control rabbits and in 12 that received the antibiotic for 4 or 8 weeks are given in Table 1.

A statistically significant loss of weight (by Student's t test, $p < 0.05$) took place withing 4 weeks in the appendix (Fig. 1) and

sacculus and in their draining nodes in the mesenteric lymphoid mass. During the second 4 weeks a further loss of weight took place in all these lymphoid organs so that by the end of 8 weeks all were

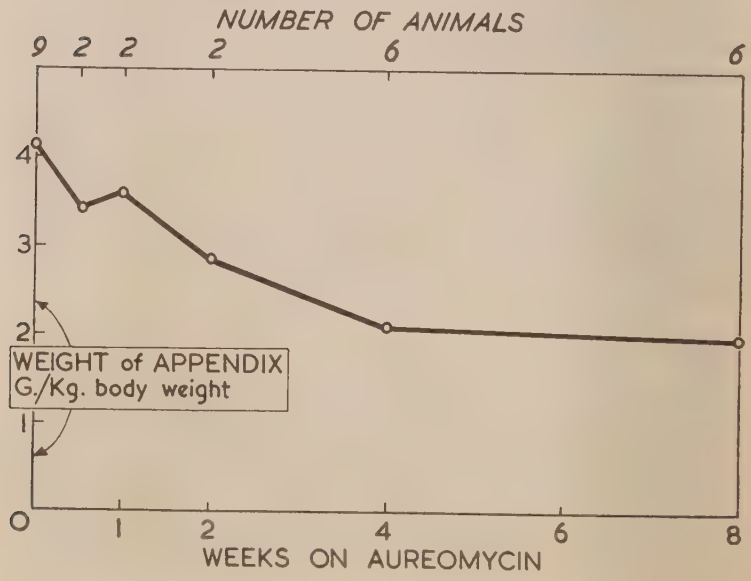
TABLE 1

MEAN WEIGHTS (G) OF VARIOUS LYMPHOID MASSES
The ileo-caecal region in control acd aureomycin treated rabbits

	Control (6 animals)	*Aureomycin treated*	
		for 4 weeks (6)	*for 8 weeks (6)*
Mean weight of group (kg)	2·2	2·0	2·2
Lymphoid mass			
Mesenteric lymphoid mass ..	1·1	0·7	0·8
Appendix	4·1	2·1	2·0
Sacculus	1·2	0·7	0·6
Proximal caecal patch ..	0·3	0·2	0·2
Terminal ileal patch	0·1	0·1	0·1

significantly reduced. None of the individual differences between the weights at the end of 4 and of 8 weeks was however significant. The probability that the reduction in the weight of the appendix

FIG. 1



could occur by chance was < 0.001 and for the sacculus and m.l.m. < 0.01. There was little change in the mean weight of the spleen during the experimental period and no significant changes in the mean weight of stomach, liver or thymus. It thus appeared that the only lymphoid masses affected were those related to the gut. Observations on stained sections confirmed these findings. There was also a slight but significant ($p<0.03$) reduction in heart weight.

Condition

The general health of the rabbits was unaffected by the treatment with aureomycin. They gained weight normally (170 g/rabbit/week.) No anaemia, change in mean red corpuscular diameter or in total or differential white count occurred. The means of the blood pyruvate estimations were very similar in treated and control animals.

Stained Faecal Smears

Direct observation of stained smears from the ileo-caecal region showed that the micro-flora was much reduced in the two treated groups; each scored a mean of 1.2 marks against 2.8 marks in the control groups. The other bacteriological observations did not reveal any significant differences between the groups.

A further series of six rabbits received a similar administration of aureomycin for shorter periods. A comparable fall took place in the weights of the lymphoid masses and those of the appendix and sacculus were already reduced as soon as 3 days after administration commenced (Table 2). In this experiment the heart weight was again reduced and the effect appeared early. As before, the general health of the animals was unaffected and a reduction in the alimen-

TABLE 2

MEAN WEIGHTS (G) OF VARIOUS LYMPHOID MASSES

The ileo-caecal region in rabbits treated with aureomycin for short periods compared with controls

	Control (*3 animals*)	*Aureomycin treated*		
		for 3 days (*2*)	*for 7 days* (*2*)	*for 14 days* (*2*)
Mean weight of group (kg)	*1·3*	*1·2*	*1·2*	*1·1*
Lymphoid mass				
Mesenteric lymphoid mass ..	2·0	2·1	2·0	1·7
Appendix	4·2	3·4	3·6	2·8
Sacculus	0·9	0·6	0·6	0·8
Proximal caecal patch ..	0·3	0·3	0·1	0·2
Terminal ileal patch	0·2	0·2	0·1	0·2

tary flora occurred during the second week (controls, 3.2 marks; 1st week 2.9 marks; second week 0.75 marks).

In normal control rabbits the appendix/spleen weight ratio is directly related to the weight of the spleen itself ($r = -0.7$, $0.01 > p > 0.001$ for 16 d.o.f).

The lymphoid aggregates in the wall of the bowel and their closely associated lymph nodes (Figs. 2 to 5) normally contain bacteria whereas the spleen and thymus normally contain lymphocytes but no bacteria. In some of these organs demonstrable bacteria are more numerous than in others. Thus in the appendix they are always present in large numbers (Fig. 2) and are also frequent in the sacculus (Fig. 3). The p.c.p. (Fig. 4) and the t.i.p. both have rather fewer still. Values of t for 10 degrees of freedom of the differences in weight induced by one month's administration of aureomycin are for appendix, 9.7, sacculus, 4.4, p.c.p. 3.4 and t.i.p. 2.3 and thus fall into the same descending order as the degree of naturally occurring bacterial infiltration. The amount of change in the m.l.m., in which only occasional bacteria are seen (Fig. 5) is of the same order as in the t.i.p.

DISCUSSION

These results show that in rabbits the weight of the lymphocyte-bearing organs in the alimentary canal is reduced by the oral administration of aureomycin. The interpretation placed upon these findings is that lymphoid tissues of the kind usually found in the rabbit's gut depends for a large part of its considerable bulk upon local stimuli derived from the presence of bacteria within it. This interpretation involves several assumptions, the validity of which may severally be considered.

The statistical calculations have been based on whole organ weights, since lymphocytes make up most of the bulk of all lymphoid organs with the exception of the spleen. In the appendix, for example, the mean thickness of the serosa, muscularis and mucosa combined averages less than 1/6th of the submucosa and similar ratios apply to other parts of the gut. (Enticknap, 1953). The thymus and mesenteric lymphoid mass are, apart from a few collagenous trabeculae and mononuclear cells, composed entirely of lymphocytes. The spleen, on the other hand, contains such a variable proportion of blood that unless the animal is bled to death its weight is a poor guide to its lymphocyte content. Thus in 9 control animals that were bled to death there was quite good correlation of spleen weight with body weight whereas none was apparent in those that were not exsanguinated. In the non-lymphoid organs, liver and stomach, no significant changes occurred. It may, therefore, be inferred that the observed variations in organ weight were in fact due to variations in lymphoid content. The one contrary observation was that of a fall in heart weight over the first month but this effect was not present at the end of the second month.

Calculations have all been based on figures for lymphoid organs expressed as g/kg body weight. While this minimises the effect of minor variations in body weight, major differences would still be important, for old animals have relatively less lymphoid tissue than young ones. In the experiments with litter mates all the groups were similar in weight and sex and identical in age composition. Calculations of appendix/spleen weight ratios emphasise the changes that occur but this ratio is itself related to splenic weight and so probably depends largely on variations in splenic blood count. Organ/body weight ratios are therefore the best available measure, provided they are interpreted with care.

It has been assumed that administration of aureomycin for a prolonged period resulted in a persistent reduction of alimentary micro-flora. This was conclusively demonstrated by direct observation of luminal and mural bacteria but not by quantitative cultures, which produced irregular results. It has been repeatedly stressed that direct observation is superior to culture in this field (Baker, Nasr, Morrice and Bruce, 1950) and it is in fact capable of producing quantitative results quite as precise as those obtainable with other material (Collard, 1952; personal, unpublished observations).

Since atrophy of lymphoid tissue has been observed at the same time and in the same regions as a reduction in bacteria, we may consider whether or not this is mere coincidence. The former was seen only in those organs that normally contain bacteria and furthermore the degree of atrophy was related to the density of the bacterial population normally seen in sections of them. The number of bacteria is in turn closely related to the bulk of lymphoid tissue in the organs. It is therefore reasonable to assume that clearance of bacteria from the lymphoid tissues caused them to atrophy and this raises the question of whether all normal lymphoid tissues are dependent upon local stimuli derived from bacteria. Spleen and thymus clearly were not, so we may consider whether they invalidate the hypothesis. Spleen/body weight ratios are rather variable and are considerably influenced by variations in blood content. The low ratio in the rabbit magnified these variations. In these experiments no constant pattern of changes was seen, but for the reasons given this need not be considered a conclusive objection. Thymus, on the other hand, is seen in histological sections to be almost entirely composed of small round cells, but some observers think these are not true lymphocytes and call them thymocytes. They disappear from the gland long before atrophy of lymphoid tissue generally occurs in the process of ageing, they have some endocrine effects, at least in rats, they give rise to tumours different from those arising from other lymphoid tissues, and in man they are in some way related to the pathological condition of myasthenia gravis. It is clear, therefore, that since the thymus differs in so may ways from alimentary aggregates, its different behaviour in these experiments does not provide any good reason for rejecting the hypothesis.

CONCLUSIONS

Administration of aureomycin to rabbits reduced the amount of bacterial infiltration normally present in the intestinal lymphoid tissue.

This reduction in bacteria was accompanied by a marked reduction in the bulk of the lymphoid tissue. Spleen and thymus were not affected.

The intestinal wall of the rabbit contains about 10 times as much lymphoid tissue, weight for weight, as that of man, and it is suggested that the stimuli provided by the intense bacterial infiltration in the rabbit are responsible for this.

REFERENCES

Baker, F., Nasr, H., Morrice, F. and Bruce, J. (1950). *J. Path. Bact.*, **62**, 617.
Bizzozero, G. (1885). *Arch. Sci. med.*, **9**, 481.
Buirge, R. E., Dennis, C., Varco R. L., and Wangensteen, O. H. (1940). *Arch. Path.*, **30**, 481.
Collard, P. J. (1952). *Lancet* ii, 843.
Digby, K. H. (1950). *Ann. roy. Coll. Surg.* (*Eng.*), **7**, 398.
Digby, K. H., and Enticknap, J. B. (1954). *Brit. J. exp. Path.*, **35**, 294.
Enticknap, J. B. (1953). *J. comp. Path.*, **63**, 7.

[*Received for publication, September 12th*, 1955]

LEGENDS TO FIGURES

Fig. 1. Chart showing effect of aureomycin on appendicular weight.

Fig. 2. Appendicular submucosa of normal rabbit showing representative number of bacteria. Gram's stain x770.

Fig. 3. Sacculus—the same.

Fig. 4. Proximal caecal patch—the same.

Fig. 5. Mesenteric lymph node—the same.

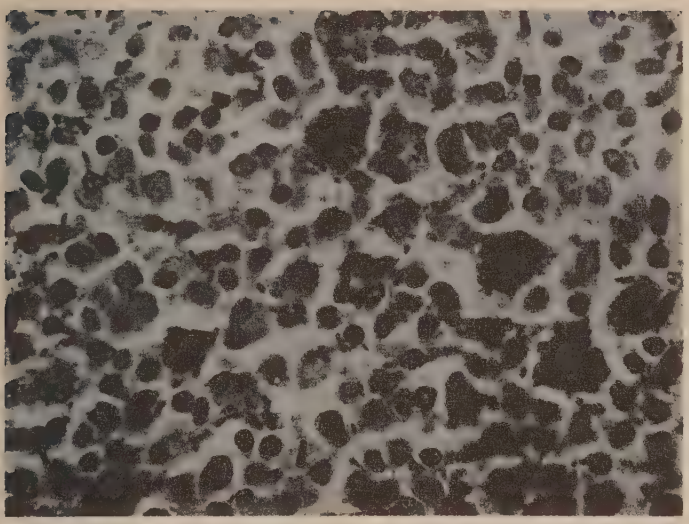


FIG. 2

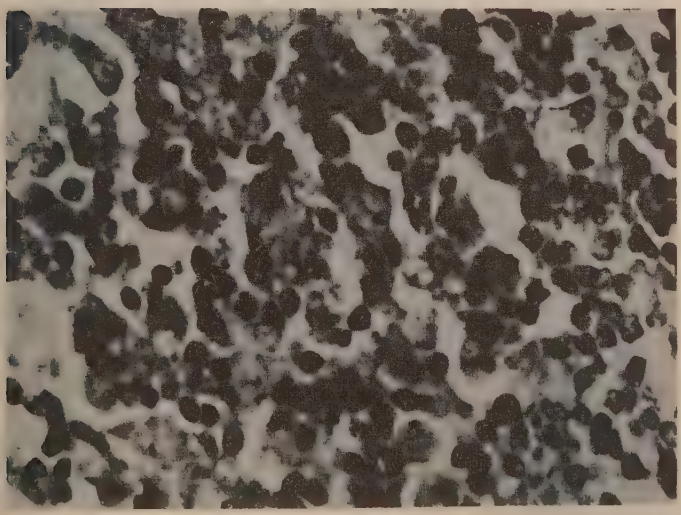


FIG. 3

To face page 108

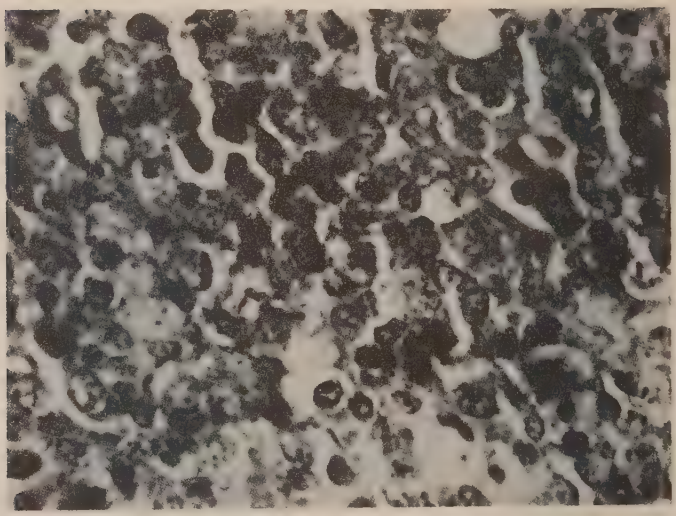


FIG. 4

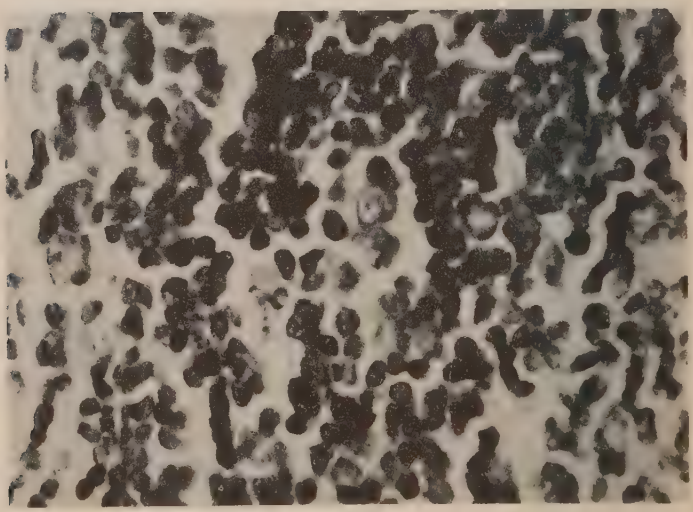


FIG. 5

EOSINOPHILIC MYOSITIS IN THE DOG

By
H. P. Harding
Department of Veterinary Pathology, University of Liverpool
and
L. N. Owen
The Veterinary Hospital, University of Liverpool

INTRODUCTION

Eosinophilic myositis was first recorded by Kuscher (1940) in Vienna and has subsequently been reported in Germany by Wirth (1941), Ullrich (1950) and Bassett (1954); in Holland by Verlinde and Winsser (1946) and Veenendaal and Ernst en Sikar (1948); in Norway by Erichsen (1952); in America by Winter and Stevenson (1952) and Moon and Wood (1954); in the Dutch East Indies by Darman and Ernst en Sikar (1952) and in England by Harding and Owen (1955). The largest number of recorded cases have been in Germany. All but two were in Alsatians, the exceptions being a miniature poodle (clinical diagnosis only) and a St. Bernard (confirmed by haematological examination).

The disease usually presents a very characterisitic clinical picture. There is bilateral swelling of the muscles of mastication, a variable degree of exophthalmos and the mouth may be held slightly open. Attacks last from about 10 to 21 days and are accompanied by a marked eosinophilia. The swelling of the muscles is followed by marked atrophy and fibrosis. Muscles other than those of mastication are rarely involved. Macroscopically affected muscles show light coloured areas intermingled with congestion and haemorrhages: microscopically, cellular infiltration is one of the most prominent features.

METHODS

Six Alsatians and a Bedlington terrier suffering from the disease have been examined. The clinical findings are recorded and in three cases where euthanasia was performed the gross and histo-pathological post-mortem findings are described.

RESULTS

Clinical Findings

Professional advice was sought by the owners because of the swelling of the dogs' heads involving principally the masseter and temporalis muscles. In most instances the muscles were soft and doughy on palpation, though in case 6 they were quite hard. In many of the cases there was also a degree of exophthalmos which was generally more marked on one side than the other; the protrusion of the membrana nictitans which accompanied the exophthalmos also

varied. There was a mild conjunctivitis, and drying and ulceration of the membrana nictitans occurred during those attacks in which the dog was unable to close its eyes fully. In three cases there was no impairment of vision during the attacks, but four dogs appeared to be blind, with no pupil reflexes in one or both eyes. In two cases the blindness persisted. There was swelling and congestion of the tonsils. In two cases attacks were accompanied by enlargement and hardness of the abdomen associated with marked splenomegaly. In two dogs (Cases 5 and 6), both of which suffered from repeated attacks, there was swelling and hardness in the limb muscles in addition to the typical changes in the head. As a rule there was little difficulty in swallowing, though there was an appreciable reduction in the muscular power of the jaws. In some instances, however, there was obvious swelling of the parotid and pharyngeal regions, leading to difficulty in swallowing. In one case with a severe attack (Case 6) the swelling was sufficient to cause some respiratory embarrassment and in this dog an intermittent purulent nasal discharge occurred.

In the intervals between attacks the muscles of the head showed atrophy, leading to a very wasted appearance and prominence of the zygomatic arch. The degree of atrophy appeared to increase progressively with succeeding attacks.

Blood samples taken during attacks always showed an eosinophilia. The lowest figure for eosinophils was 10 per cent. (2,900 per cu.mm.), and the highest 62 per cent (11,460 per cu.mm.), the full figures being given in Table 1. Although the dogs improved clinically after periods varying from a few days up to one month, the eosinophilia usually persisted for some time afterwards, but ultimately fell to a normal level (Case 2). The interval between attacks varied greatly, from approximately 12 months (Cases 2 and 7) to a few weeks (Case 5) and there was also a marked variation in the severity of attacks, even in the same animal. In two dogs (Cases 1 and 2) there was a slight rise in temperature (103.2°F.,) but the others showed normal temperatures throughout.

Bile pigments were demonstrated in urine samples from four of the dogs.

Post-mortem Findings

Three dogs were destroyed and autopsied. In Case 7 the examination was performed by Miss M. Dalby, only the histopathology being carried out by one of the authors.

Macroscopic Lesions (Cases 1 and 3).

The masseter and temporalis muscles were enlarged, the swelling being doughy in character. They were dark in colour and showed haemorrhages and numerous focal or elongated pale areas (Fig. 3). The eyes were prominent though the eyeballs themselves appeared to be of normal size. The submaxillary lymph nodes were enlarged and inflamed. The spleen showed gross enlargement. No significant

lesions were observed in any of the other muscles or organs. Blocks of tissue were taken and fixed in formol-mercury saline for histological examination.

TABLE 1

TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNT OF DOGS CLINICALLY AFFECTED WITH EOSINOPHILIC MYOSITIS

	Leucocytes per cu.mm.	*Eosinophils per cu.mm.*	*Neutrophils per cent.*	*Lymphocytes per cent.*	*Monocytes per cent.*	*Eosinophils per cent.*
Case 1.						
August 1954	26,000	9,360	54	9	2	36
February 1955	25,700	10,280	49	7	4	40
Case 2. (*Second attack*)						
1.3.55.	16,600	7,698	46	5	1	48
2.3.55.	23,200	10,556	45·5	6·5	2·5	45·5
3.5.55.	18,500	11,460	27·5	7·5	3·0	62·0[1]
4.3.55.	18,000	7,830	45·0	6·0	5·5	43·5[2]
5.3.55.	20,000	11,500	32·5	5·5	4·5	57·5
10.3.55.	14,100	4,371	40·0	22	5	31
19.3.55.	11,300	1,017	74·5	11·5	5	9[3]
12.8.55.	10,200	2,040	62·0	15	3	20
19.8.55.	9,300	465	64·0	26	5	5
		1. Muscle biopsy performed. 2. Clinical improvement observed. 3. Weekly blood counts were carried out during the ensuing period of five months, eosinophils varying between 2 and 5 per cent.				
Case 2. (*Third attack*)						
21.10.55.	16,650	7,326	46	5	5	44[1]
22.10.55.	17,700	5,664	55	7	6	32[2]
26.10.55.	11,000	4,730	43	8	6	43[3]
		1. First day of attack. 2. Clinically normal, following injection of ACTH. 3. Still clinically normal.				
Case 3.						
16.4.55.	15,250	1,830	76	9	3	12
26.4.55.	29,000	2,900	74	12	4	10
Case 4.						
19.7.55.	18,400	8,830	38	11·5	2·5	48
22.7.55.	20,000	11,400	27·5	13·5	2·0	57
Case 5.						
1.7.55.	10,650	2,236	61	14	4	21
10.8.55.	17,800	6,942	46	11	4	39
Case 6.	10,300	1,957	61	15·5	4·5	19

Microscopic Lesions

Masseter, temporalis and pterygoideus muscles. These groups showed very severe changes, though there was marked variation even from field to field in the same section. Many of the muscle fibres appeared normal; others showed longitudinal fibrillation and some had under-

gone necrosis and appeared hyaline (Fig. 4). In some areas the fibres were separated only by slight oedema and cellular infiltration: in others the degree of infiltration led to wide separation of the fibres, (Fig. 5) and in the areas most severely affected only isolated fibres or fragments persisted in the middle of masses of cells, sometimes accompanied by haemorrhage (Fig. 6). The proportions of the infiltrating cells also varied widely. In general eosinophils made up at least 70 per cent. of the total, with macrophages and plasma cells accounting for almost all the rest. Giant cells which were seen quite commonly, appeared to be derived from the destroyed muscle fibres by a condensation of the sarcolemma nuclei. Chains of up to about six sarcolemma nuclei were also observed. There were a number of foci consisting almost entirely of eosinophils, many of the cells showing necrosis. There were also many focal areas in which plasma cells greatly predominated, some appearing to surround small arteries. Blood vessels in general were dilated, and showed endothelial swelling.

Other muscles. Mild changes, involving hyaline degeneration of some of the fibres with minimal cellular infiltration were noted in the abdominal wall, intercostals and biceps brachii. Marked changes with focal or diffuse cellular infiltration of the endomysium and perimysium, necrosis of muscle fibres sometimes with haemorrhage were seen in the occipito-mandibularis, sterno-hyoideus, sterno-cephalicus, biceps femoris, triceps brachii, pectoralis major, gracilis, and rectus abdominis.

In muscles where the cellular reaction was only focal it was sometimes necessary to examine several blocks of tissue to detect the lesions.

Tongue. The tip showed hyaline muscle fibres; in addition, slight cellular infiltration and thrombosis of the arteries was observed in Cases 1 and 7. Towards the root of the tongue lesions were severe, with the typical infiltration and destruction of muscle. Foci of cells (chiefly plasma cells) also occurred in the fat and the subepithelial tissues (Fig. 7).

Oesophagus (Case 7). Marked cellular infiltration was observed in the muscular layer, in the submucous connective tissue and in the loose connective tissue surrounding the oesophagus.

Tonsil. In Case 3 the lymphoid follicles were very active and there was slight infiltration of the associated mucous glands with lymphocytes. In Case 7 the tonsil was very congested and there was some haemorrhage. The epithelium was infiltrated with neutrophils, and the sinuses were packed with plasma cells, necrobiotic neutrophils and eosinophils. The mucous glands showed plasma cell infiltration.

Submaxillary lymph nodes. The loose connective tissue surrounding the nodes sometimes showed haemorrhage and leucocytic infiltration, principally eosinophils and plasma cells. The nodes were congested

and oedematous, and in some cases the sinuses contained numerous red cells and a fibrinous exudate. Most of the sinuses were packed with cells with a preponderance of plasmocytes, eosinophils being less numerous. Many macrophages were observed, a large proportion of them being full of granules of haemosiderin.

Submaxillary salivary gland. This was congested and showed a slight diffuse cellular infiltration with definite foci round some of the smaller arteries and ducts. Lymphocytes and plasma cells predominated, only about 25 per cent. being eosinophils.

Liver. The hepatic cells showed cloudy swelling and slight fatty change. The sinusoids were congested. In all cases there was a cellular infiltration round branches of the hepatic vein, giving rise to perivascular cuffing. In Case 3 this cuffing also occurred round some of the centrilobular veins, and in Case 7 there was, in addition, some infiltration of the portal tracts.

Spleen. This was extremely congested. The reticulum cells contained haemosiderin, the sinusoids contained numerous plasma cells. In Case 3 numerous cells were observed resembling eosinophilic myelocytes, but which were probably necrobiescent eosinophils.

Brain. In Case 1 (which showed blindness) lesions were observed in the corpora quadrigemina. There was perivascular cuffing and focal gliosis and many of the large neurones showed chromatolysis and satellitosis.

No significant lesions were observed in any of the other tissues which were examined. As a general rule those animals which were destroyed during the early phases of an attack showed a preponderance of eosinophils in the lesions, whereas in the later stages plasma cells and macrophages formed a significant proportion of the whole.

Diagnostic Investigations

The greater part of this work was carried out on Case 2, a five and a half year old Alsatian bitch, seen during the second attack of the disease, the first having occurred six months previously.

A blood count showed 16,600 leucocytes/cu.mm. and 48 per cent. eosinophils. Blood urea, blood non-protein nitrogen, blood sugar, plasma sodium and plasma potassium fell within normal limits. The urine contained bile pigments. Bacteriological examination of tonsillar and nasal swabs revealed no organisms of pathological significance.

On the 9th day of the attack anaesthesia was induced with thiopentone sodium and biopsy specimens were obtained from the masseter and biceps femoris muscles. The masseter showed the characteristic eosinophilic infiltration. The biceps femoris was submitted to parasitological examination, and proved completely negative. Recovery from the anaesthetic was uneventful. By the following day the temperature had fallen to normal, and rapid regression of the muscle swelling commenced, the exophthalmos

having virtually disappeared. Muscular wasting was apparent in a further three days. At the time of operation the blood count showed 18,500 leucocytes per cu.mm., eosinophils 62 per cent; the following day the figures were 18,000 leucocytes per cu.mm., eosinophils 43 per cent. On the second post-operative day they were 20,000 leucocytes per cu.mm., eosinophils 57 per cent. There was then a progressive fall in the total and eosinophil counts, as shown in Table1, reaching a normal level in twelve days.

The dramatic clinical improvement, in which the eosinophil count had fallen from 11,470 per cu.mm. to 7,740 per cu.mm. in less than 24 hours, a reduction of about 34 per cent., appeared to stem from the anaesthesia and operation. This observation led to speculation as to the possible mechanism of the response. It is known that stress results in stimulation of the adrenal cortex, and that the injection of the adrenocorticotrophic hormone (ACTH) into normal dogs results in an eosinopaenia (Marten, Skillen and Deubler, 1954). Some effect on the adrenal gland was therefore considered as a possible explanation.

Marten, et al. (1954) considered that the injection of between 10 and 50 i.u. of ACTH into normal dogs should produce a decrease of at least 70 per cent. in the circulating eosinophils within seven hours, and suggested that a fall of less than 70 per cent. indicated adrenocortical insufficiency. They pointed out, however, that under certain circumstances (e.g. allergy) the production of eosinophils may exceed the ability of ACTH to destroy them. A lesser effect can be produced by other agents which indirectly stimulate the adrenal cortex, e.g. adrenalin.

The following tests were carried out to determine whether the adrenal cortices of this dog were functioning normally.

ACTH. 20 i.u. were injected intramuscularly. The following results were obtained:— before injection, 326 eosinophils per cu.mm; five hours after injection, 3 eosinophils per cu.mm., a percentage fall of 97.

Adrenalin. 1 ml. of 1/1000 liquor-adrenalin hydrochloride was injected subcutaneously on a separate occasion. The results were as follows:— before injection, 1,017 eosinophils per cu.mm; four and a half hours after injection, 617 eosinophils per cu.mm., a percentage fall of 39.3.

Allergic State

As one of the main features of this disease is an eosinophilia it seems possible that the cause may be related to an allergic condition. In order to determine whether this bitch was allergic to any of the commoner antigenic substances she was tested with "Bencard" allergens by the standard intradermal technique. The following were used:— bacteria, 18 species; moulds and fungi, 16 species; foods, 38 varieties (including cow's milk); hairs and danders, 11 types; miscellaneous, soil, dust, serum and histamine. In addition two

groups of hormones were tried:— Compound Group H.1, contained desoxycorticosterone acetate, oestradiol benzoate, oestrone, progesterone, stilboestrol, testosterone propionate; Compound Group H.2, contained aqueous extracts of ovary, pituitary (anterior lobe), testis and thyroid.

A doubtful reaction was produced by the cow's milk allergen. Strong positive reactions were obtained with histamine and H.2. Subsequent testing with the individual constituents of H.2 showed only a weak positive reaction to the ovary extract.

The hormone antigens were injected into Case 6, a six year old Alsatian bitch, on the 21st day of an attack, and identical results were obtained. In Case 5, an Alsatian dog, there was, however, no reaction to any of the injections.

The antigens were tested on the following normal dogs—one mongrel terrier, three Alsatian dogs and two mongrel terrier bitches. The only reaction was a slight erythema to H.2 in one of the bitches.

Therapeutics

Attempts which have been made to treat this disease have been summarised by Winter and Stephenson (1952). Antihistamines were an obvious choice on the assumption that the condition was a true allergy (Wirth, 1941), but Ullrich (1950) achieved no results with Antisin (Chlorcyclizine), and four of our cases received various antihistaminics without appreciable benefit. It thus appears unlikely that the condition can be associated with histamine release, though the possibility that it is an allergy cannot be excluded. Treatment with various antibiotics, aspirin and vitamins were tried on a number of our cases with no obvious beneficial effects.

Moon and Wood (1954) described the treatment of a case with cortisone, which was used because of its effect in reducing the level of circulating eosinophils. They gave 50 mg. of cortisone intramuscularly on the fourth day of an attack and noted an improvement within 24 hours: moreover, 650 mg. were given over the next 14 days with further marked improvement. During this time the eosinophil count fell from 22.5 per cent. to 13 per cent.

Case 5, a three and a half year old male Alsatian, had been suffering for two years from regular attacks, the interval varying between five and twelve weeks. Various treatments had been tried, but none of them had any significant effect on the duration or severity of the attacks. On the next attack ACTH was used in the following dosage:—30 i.u. intramuscularly as soon as the muscle swelling was observed, 30 i.u. on the third day, 20 i.u. on the fifth day and 20 i.u. on the seventh day. After the first injection the head swelling regressed within a few hours, and although the head started to swell again, subsequent injections brought about a remission of the symptoms. By the eighth day the attack had been completely arrested, and for the first time the dog appeared to have suffered no discomfort during an attack. Subsequently, attacks commenced at much shorter

intervals than before, but an injection of ACTH caused complete remission of the symptoms within three hours and the dog appeared to remain in excellent spirits. A comparison was made between cortisone and ACTH. It was found that although cortisone seemed to prevent the muscle swelling from reaching the usual proportions, it did not avert it completely, and did not prevent systemic disturbances during the attack. After numerous attacks which responded rapidly to injections of ACTH, a very severe bout developed in which the dog appeared to feel choked, was unable to breathe or swallow properly and was in extreme discomfort, owing apparently to an affection of all the skeletal muscles. This attack lasted for a number of days. It finally resolved after treatment with 25 i.u. of ACTH twice daily and sedatives to relieve the pain. Since this severe attack the dog has remained free of symptoms for several months although the eosinophil level has remained high.

ACTH was also used for treating the third attack suffered by Case 2. The injection was given on the day that marked swelling of the head was observed and in three hours the dog appeared to be almost normal. The head swelling had regressed and there was no impairment of activity or other bodily functions.

Blood examinations carried out during the course of treatment revealed that despite the very marked clinical improvement, there was only a slight fall in the level of circulating eosinophils.

Haematological Examinations

Although full blood examinations were carried out, only the data relating to the leucocytes are given as, apart from some degree of polychromasia during the attacks, all the figures relating to the

TABLE 2

TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNT OF ALSATIANS RELATED TO CLINICAL CASES

Dog	*Leucocytes per cu.mm.*	*Eosinophils per cu.mm.*	*Neutrophils per cent.*	*Lymphocytes per cent.*	*Monocytes per cent.*	*Eosinophils per cent.*
Sons and daughters of Case 1.						
1 (M)	10,500	1,995	49	27	5	19
2 (F)	12,300	1,230	70	16	4	10
3 (F)	11,250	2,700	56	15	4	24
4 (F)	9,500	1,425	60	20	5	15
5 (M)	12,000	3,360	49	18	5	28
6 (M)	10,500	630	78	12	4	6
		Eosinophil mean value 17 ± 8.34 per cent.				
Relations of Case 5.						
1 (M)	6,200	310	66	25	4	5
2 (F)	5,900	472	72	17	3	8
3 (F)	8,250	907.5	62	22	5	11
Daughter of Case 6.						
1	14,000	1,120	59	20.5	2.5	8

erythrocytes fell within the normal ranges in every case. The complete white cell counts of the clinical cases are given in Table 1, and show a range during the attacks of 10 to 62 per cent. eosinophils (1,830 to 11,500/cu.mm.). The cells were almost entirely typical bilobed mature forms except during the third attack in Case 2 when numerous eosinophilic myelocytes were observed. The eosinophils observed in the post-mortem material were almost all mature cells.

In order to determine whether there might be any familial tendency towards eosinophilia (a condition recognised in human medicine) six clinically normal progeny of Case 1 were examined (Table 2). The mean eosinophil count was found to be 17.0 ±8.34 per cent. which, when analysed statistically, is significantly higher than that in clinically normal unrelated Alsatians (Table 3). Five of the six were from two bitches themselves related to Case 1, whereas the dog with the 6 per cent, eosinophil count was out of an unrelated bitch.

TABLE 3

TOTAL AND DIFFERENTIAL WHITE CELL COUNT OF CLINICALLY NORMAL ALSATIANS

No.	*Leucocytes per cu.mm.*	*Eosinophils per cu.mm.*	*Neutrophils per cent.*	*Lymphocytes per cent.*	*Monocytes per cent.*	*Eosinophils per cent.*
1.	10,400	156	66	22.5	10.5	1.5
2.	11,200	224	69	23	6	2
3.	9,700	291	73	18	6	3
4.	12,500	375	70	14	10	3
5.	13,600	408	64	27	6	3
6.	11,600	348	60	28	8	3
7.	12,900	516	76	16	4	4
8.	11,000	550	67	24	4	5
9.	11,600	580	55	33	7	5
10.	12,000	840	63	23	7	7
11.	10,900	763	64	24	5	7
12.	10,700	749	65	22	6	7
13.	12,850	1,028	64	22	6	8
14	5,150	412	69	19	4	8
15.	8,800	792	70	17	4	9
16.	12,000	1,080	59	22	10	9
17	10,600	1,272	67	14	7	12
		Eosinophil mean value 5.68 ±2.97 *per cent.*				

Three dogs related to Case 5 and one to Case 6 had eosinophil counts which did not differ significantly from those given in Table 3, (unrelated, clinically normal Alsatians) which show a mean of 5.68, with a standard deviation of ±2.97 per cent. eosinophils. The figures for 27 Alsatians with various diseases (Table 4) are 6.04 ±4.81 per cent. These figures may be of some interest as widely divergent figures for the eosinophil levels in dogs are given. Boddie (1950) quotes 2.0 ± 2 per cent., Gaiger and Davies (1955) 9.0 per cent. and Coffin (1947) states that the level in dogs fluctuates widely,

giving figures which could be interpreted as indicating a range of 0.5 to 40 per cent.

TABLE 4.

TOTAL AND DIFFERENTIAL WHITE CELL COUNT OF ALSATIANS WITH VARIOUS DISEASES

Disease	*Leucocytes per cu.mm.*	*Eosinophils per cu.mm.*	*Neutrophils per cent.*	*Lymphocytes per cent.*	*Monocytes per cent.*	*Eosinophils per cent.*
1. Distemper	7,150	214	57	23	17	3
2. Distemper	16,750	670	62	31	3	4
3. Distemper	16,600	0	59	24	17	0
4. Distemper	20,600	0	85	12	3	0
5. Distemper	19,550	0	85	8	7	0
6. Distemper	6,400	384	67	20	7	6
7. Distemper	10,750	107	70	15	14	1
8. Chronic colitis	6,000	840	66	17	3	14
9. Enteritis	15,500	1,550	67	14	9	10
10. Enteritis	7,500	675	68	19	4	9
11. Distemper & Taeniasis	10,000	1,800	59	18	5	18
12. Metritis	11,050	552	82	9	4	5
13. Chronic nephritis	13,900	417	85	6	6	3
14. Abdominal neoplasia	18,050	1,083	70	9	15	6
15. Taeniasis	9,150	366	58	34	4	4
16. Conjunctivitis	8,350	501	65	25	6	6
17. Severe orbital eczema	10,350	1,345	61	19	7	13
18. Blepharitis	8,200	1,148	67	15	4	14
19. Staphyloma	10,350	310	63	31	2	3
20. External otitis	16,000	800	67	25	3	5
21. Demodectic mange	15,200	1,064	37	49	7	7

TABLE 4—*Continued*

Disease	*Leucocytes per cu.mm.*	*Eosinophils per cu.mm.*	*Neutrophils per cent.*	*Lymphocytes per cent.*	*Monocytes per cent.*	*Eosinophils per cent.*
22. Anal furunculosis	11,250	337	76	16	5	3
23. Anal furunculosis	14,600	292	87	7	3	2
24. Anal abscess	19,550	195	79	18	2	1
25. Chronic abscess of foot	14,500	870	60	29	5	6
26. Intramedullary pinning. 4 weeks	14,000	1,260	66	18	7	9
27. Toxaemia of unknown origin	14,500	1,595	65	19	5	11

Eosinophil mean value 6.04 ± 4.81 *per cent.*

DISCUSSION

From our observations and those of other authors there are clearly differences in the manifestations of eosinophilic myositis, though the syndrome as a whole is easily recognisable. Ullrich (1950) stated that a definite diagnosis of the condition could only be made by a blood examination and gave figures which showed that in all but one of his cases the eosinophil levels were between 12 and 20 per cent. However, in 50 Alsatians which had never suffered from eosinophilic myositis, we found 11 with eosinophil levels between 10 and 28 per cent, so that a blood examination alone might prove deceptive. Though for all practical purposes the clinical picture in conjunction with a blood count should be sufficient to permit a diagnosis, the only certain method of confirming the disease would appear to be by muscle biopsy.

During attacks it was possible to open the jaws to their full extent, although it appeared to cause pain, probably due to passive extension of the affected muscles (Ullrich, 1950). In no case did the affected muscles feel hot and there was little reaction to deep palpation. There is invariably some atrophy of the affected muscles following an attack, so that the wasting becomes progressively more marked with each attack, and the eyes ultimately become very sunken. However, despite histological evidence of fibrous replacement of muscle tissue, there was no evidence that the jaws ever become immobilised. Otten (1952) described a case which had numerous attacks, and Case 5 in the series reported in this paper had almost 30 attacks. Nevertheless there is no record of any animal having actually died from the disease.

A disease which might cause some confusion in diagnosis is fibromyositis of the jaws. The latter does not appear to show any breed predilection and results in almost complete immobilisation of the mandible, but during its early phases there may be an eosinophilia and a cellular infiltration of the muscles of mastication (Head, 1955). We have seen one case in a Red Setter in which persistent manipulation of the mandible ultimately restored the full range of movement, but severe muscular atrophy persisted and would have been indistinguishable from the quiescent stage of eosinophilic myositis.

All the cases described in the literature have involved the muscles of mastication only, with the exception of an 18 months old Alsatian which had a severe attack accompanied by stiffness of the legs followed by atrophy of the biceps femoris and triceps brachii muscles (Darman and Ernst en Sikar, 1952). In our series of cases there were several attacks in which swelling of the limb muscles occurred, and swelling and hardness of the abdomen were also observed. From our histological findings it would appear that almost all the voluntary muscles of the body, including the tongue and oesophagus, may be affected to a greater or lesser degree, so that the previous conception of the disease as a local myositis is inadequate. We were, however, unable to explain the more severe reaction in the muscles of mastication. Cellular infiltration of the liver occurred in all the dogs examined and is described for the first time. Splenomegaly was present in the three dogs submitted to post-mortem examination, and in Case 5 the enlarged spleen was readily palpable.

The blindness in Case 1 may have been associated with the lesions in the brain, as in Case 3 in which the brain was unaffected the sight was unimpaired.

Ullrich (1950) stated that the urine was normal, but in the only four cases we examined bile pigments were present. It would appear likely that the pigments are the result of haemorrhage and muscle breakdown, the loading of reticulum cells with haemosiderin in the lymph nodes, spleen, liver and bone marrow tending to support this view. The polychromasia observed during these attacks probably indicated an active regeneration of erythrocytes, adequate in all the cases so far examined to maintain normal blood levels. No trace of jaundice was observed in any of the cases.

Despite careful observation, no factors which might have initiated the attacks could be discovered. Case 5, in which attacks were particularly frequent, was kept in close contact with and under the same conditions as a number of other Alsatians for several years, and none of them developed the condition. It would thus appear unlikely that an infectious agent can be incriminated. The only definite evidence of allergy was the strongly positive reactions to the compound allergen H.2. in two bitches. As allergy was not seen in the male (Case 6), the influence of sex may have some bearing on our observation that two of the bitches suffered attacks more or less regularly during the periods of pseudo-pregnancy.

The tests carried out on Case 6 indicated that adrenocortical function was normal, but it would seem desirable to carry out further experiments on these lines as we are unable to explain the fact that between attacks some dogs show high eosinophil levels while others fall into the normal range. In this connection, the eosinophilia found in the offspring of Case 1 is of interest.

It is very difficult to assess the efficacy of therapeutic measures, as attacks vary so much in intensity and duration. However, the effect of ACTH is so dramatic that it must be considered as of very real value. It still remains to be seen whether the increased frequency of attacks observed in Case 5 was related to the use of the drug, or whether it was coincidental.

CONCLUSIONS

Seven cases of eosinophilic myositis are described, six in Alsatians and one in a Bedlington terrier.

Blood counts of affected, related, and unrelated Alsatians indicate that high eosinophil levels may be encountered in clinically normal animals.

The aetiology and treatment are discussed. While the cause remains obscure, ACTH was found to be a promising therapeutic agent.

ACKNOWLEDGMENTS

We wish to thank G. Weston, F.I.M.L.T., for technical assistance, and the following veterinary surgeons for their co-operation: J. Adamson, Mrs. M. Clare, Miss M. Dalby, K. W. Head, A. Mutch, A. Steele-Bodger and Miss N. Wickham. We are also indebted to Messrs. C. L. Bencard Ltd. for information and a generous supply of allergens for skin testing, and to Messrs. Sharp & Dohme, West Point, Pennsylvania, for a supply of "Cortone" Acetate.

REFERENCES

Bassett, C. S. (1954). *J. Roy. Army vet. Cps.*, **25**, 3.
Boddie, G. F. (1950). *Diagnostic Methods in Veterinary Medicine*, 3rd Edn., Oliver and Boyd; London.
Coffin, D. L. (1947). *Manual of Veterinary Clinical Pathology*, Comstock Publishing Co.; Ithaca, New York.
Darman, A. J., and Ernst en Sikar, A. M. (1952). *Hemera Zoa*, **59**, 153.
Erichsen, S. (1952). *Nord. vet. Med.*, **4**, 139.
Gaiger, S. H., and Davies, G. O. (1955). *Veterinary Pathology and Bacteriology*, 4th Edn., Baillière, Tindall & Cox; London.
Harding, H. P., and Owen, L. N. (1955). *Vet. Rec.*, **67**, 460.
Head, K. W. (1955). *Personal communication.*
Kuscher, A. (1940). *Wien. tierärztl. Mschr.*, **27**, 177.
Martin, J. E., Skillen, R. G., and Deubler, M. Josephine. (1954) *Amer. J. vet. Res.*, **15**, 489.
Moon, C., and Wood, A. C. (1954). *J. Amer. vet. med. Ass.*, **125**, 312.

Otten, E. (1952). *Tierärztl. Umsch.*, **7**, 52.
Ullrich, K. (1950). *Monatsch. vet. Med.*, **7**, trans. in *J. Roy. Army vet. Cps.*, **25**, 2.
Verlinde, J. D., and Winsser, J. (1946). *Tijdschr. Diergeneesk.*, **71**, 358.
Veenendaal, H., and Ernst en Sikar, A. M. (1948). *Ibid.*, **73**, 292.
Whitney, J. C. (1955). *Vet. Rec.*, **67**, 1140.
Winter, H., and Stephenson, H. C. (1952). *Cornell Vet.*, **42**, 531.
Wirth, D. (1941). *Dtsch. tierärztl. Wschr.*, **49**, 626.

[*Received for publication, September 15th,* 1955].

LEGENDS

Fig. 1. Case 2. During attack.

Fig. 2. Case 2. Sixteen days after attack. The wasting of the temporalis and masseter muscles is clearly shown.

Fig. 3. Cross section of temporalis muscle from Case 1.

Fig. 4. Hyaline change and mild cellular infiltration of affected muscle, Case 1, H. & E. x 75.

Fig. 5. More marked cellular infiltration of muscle, Case 1, H. & E. x 160.

Fig. 6. Severely affected muscle, Case 1, H. & E. x 220.

Fig. 7. Lesion of tongue, Case 3, showing necrotic area, H. & E. x 75.

FIG. 1

FIG. 2

To face page 122

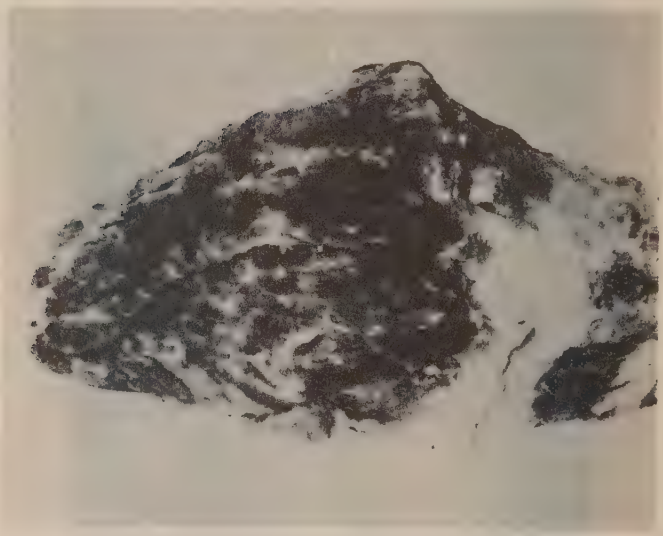


FIG. 3

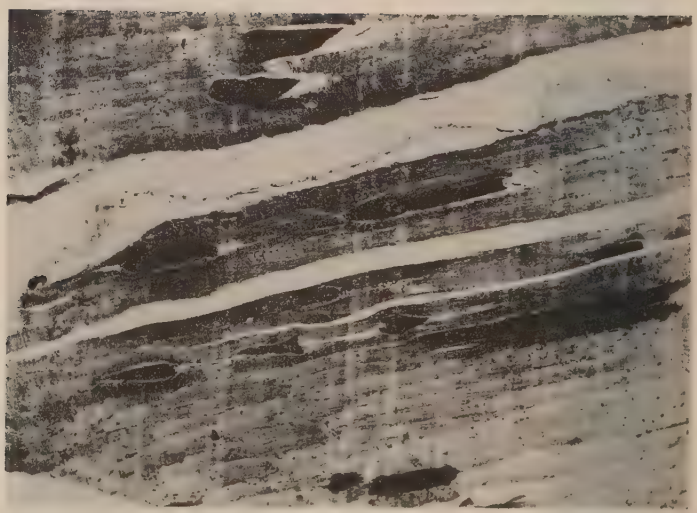


FIG. 4

FIG. 5

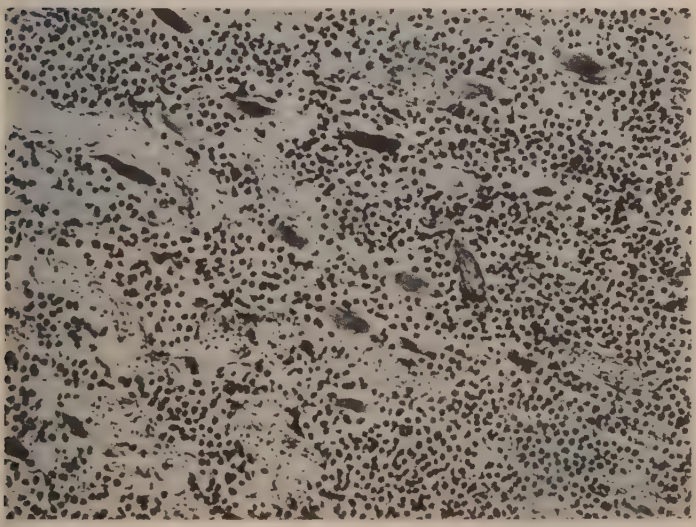


FIG. 6

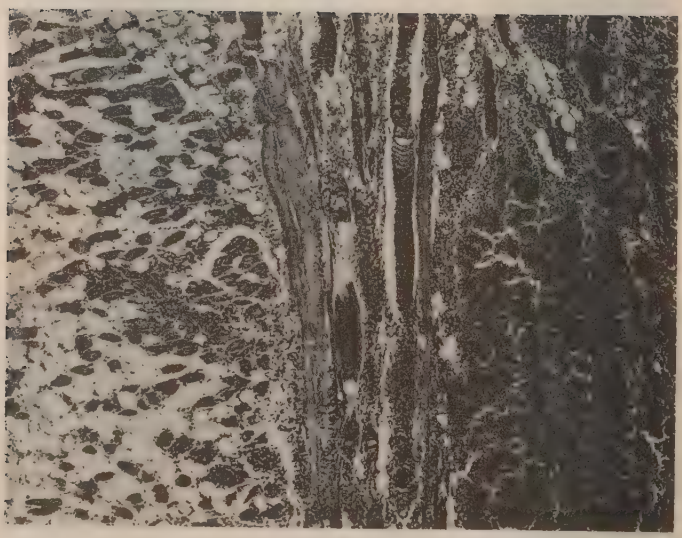


FIG. 7

NATURAL AND EXPERIMENTAL TUBERCULOSIS IN MONKEYS

With Observations on Immunization and Chemotherapy

By

J. Francis

*Imperial Chemical (Pharmaceuticals) Ltd., Wilmslow, Manchester, England**

INTRODUCTION

Information on tuberculosis in monkeys was reviewed by Francis (1956). Tuberculosis has not been recorded in monkeys living in the wild state, although this cannot be due to lack of close contact between individuals. The high susceptibility to tuberculosis of monkeys kept in captivity has however long been recognised; thus Straus (1895) reported that 43 per cent of 173 monkeys dying at the London Zoological Gardens had lesions of tuberculosis, and in two French zoological gardens the figures were 20 and 22 per cent. There have been many other reports of the high incidence of tuberculosis in captive monkeys.

Schroeder (1938) has given the best general picture of the disease. From 1930 onwards monkeys began to be imported in large numbers, perhaps 40,000 per annum, into the U.S.A., usually under very bad hygienic conditions. One zoological society received 200 *Macaca mulatta* to populate a monkey island. Within ten days of arrival 10 per cent had died and during the course of the summer another 125 monkeys died. All deaths were attributed to acute tuberculosis. From the replies to a questionnaire, Schroeder concluded that the average death rate from tuberculosis was about 10 per cent per annum and that 80 per cent of all deaths were due to tuberculosis. This is approximately the same as the mortality from tuberculosis observed in some Red Indian reservations (Dubos and Dubos, 1953).

Scott (1930) gave a detailed description of the lymphatic system of monkeys and of the macroscopic tuberculous lesions in 51 monkeys dying in the Zoological Gardens, London, over a period of three years. Fox (1923) autopsied 498 monkeys and described the tuberculous lesions that were found in 38.8 per cent. The findings of these workers are summarised in Table 1.

Cobbett and Griffith (1913) found that monkeys reacted poorly to the subcutaneous injection of tuberculin, but this test was used successfully by Fox (1923) to control tuberculosis in a monkey colony. It appears that Schroeder (1938) was the first to use the intradermal test in monkeys. He injected 0.1 ml. of a 10^{-2} dilution of P.P.D. (i.e. 1 mg.) into the upper eyelid. A positive reaction was indicated by oedema and redness which usually developed within 16 hours and persisted for 72 hours, and occasionally there was a small area of necrosis. Monkeys with early or terminal infections sometimes failed to react. Following Schroeder's work Kennard, Schroeder, Trask and Paul (1939) injected 0.1 ml. of a 10^{-2} dilution of O.T. subcutaneously into the upper eyelid of monkeys, of which the majority were *M. mulatta*. They tuberculin-tested and autopsied 110 monkeys and observed a total error in the results of the test of 10.8 per

*Present address: The Veterinary School, University of Queensland, Brisbane, Australia.

cent. Habel (1947) used a similar technique when working on the control of tuberculosis in a monkey colony: the degree of correlation between test and autopsy was 93 per cent or more in all tuberculin-positive animals no matter what the time interval from test to autopsy. The correlation was 91 per cent in the tuberculin-negative animals if autopsied within one month of being tested. He was successful in controlling the disease. More recently Skavlem (1953) had difficulty in controlling the disease by tuberculin testing and hygienic methods, but reported good results with isoniazid.

There are few descriptions or illustrations of the histological findings in naturally or artificially infected monkeys. This is surprising, as the disease in monkeys is usually considered to be of special interest from the comparative viewpoint.

The purpose of the present paper is to present such information, and to describe experiments on immunization and chemotherapy, including the action of 5-amino-7-methyl-1:2:4:6-tetra-azaindene (7438). The antituberculous action of this drug was detected in chick embryos by Francis and Hoggarth (1953). Its absorption and toxicity in mice, guinea-pigs, monkeys and dogs was described by Francis, Martin, Rose and Spinks (1954), who showed that it had good action against some types of tuberculosis in mice and guinea-pigs.

MATERIALS AND METHODS

M. mulatta monkeys imported by boat from India were studied, and were doubtless infected in India after capture or on the voyage to England. The Weybridge C strain (Francis, Spinks and Stewart, 1950) of human tubercle bacilli was used to infect normal monkeys. The P.P.D. tuberculin used was obtained from the Veterinary Laboratory, Ministry of Agriculture, Weybridge. The concentration of 7438 was estimated by a fluorimetric method (Francis, *et al.*, 1954). Wintrobe tubes were used for estimating the blood sedimentation rates and were read after 30 minutes. The post-mortem techniques, and the methods of infecting monkeys and assessing results were the same as those employed by Francis, Spinks and Stewart (1950). Briefly the method was as follows:

*Lungs (right or left)**	*Extent of change*	*Score if area consolidated*	*Score if area caseated*	*Score if area cavitated*
	Less than 1/3 of cut surface	1—2	1	1—2
	1/3 — 2/3 of cut surface	3—4	2	3—4
	More than 2/3 of cut surface	5—6	3	5—6

Bronchial lymph-nodes (right or left)	*Degree of enlargement*	*Score*	*Proportion of caseation*	*Score*	*Proportion of cavitation*	*Score*
	Slight	1	Less than 1/3 of cut surface	1	Less than 1/3 of cut surface	1—2
	Moderate	2	1/3—2/3 of cut surface	2	1/3—2/3 of cut surface	3—4
	Gross	3	More than 2/3 of cut surface	3	More than 2/3 of cut surface	5—6

*Scores were additive, as shown below: more than 2/3 of cut surface affected, 5; 1/2—2/3 of cut surface caseous, 2; less than 1/3 of cut surface cavitated, 1; total score 8.

Lesions in other organs were assessed in a similar manner.

The number of tubercle bacilli in microscopic sections was assessed as follows:

1. Scanty: difficult to find
2. Isolated bacilli found easily
3. Small clumps found easily or fairly numerous isolated bacilli

} As seen in animals included in Groups 1 and 2

4. Numerous small clumps and isolated bacilli
5. Numerous large clumps in fields

} As seen in fowls and infection of mice with human, bovine and avian types of tubercle bacilli

6. Masses of bacilli — As seen in severe infections with leprosy bacilli, and vole bacilli in mice.

RESULTS

Twenty naturally infected monkeys were examined; 15 reacted to the intrapalpebral injection of 10^{-2} mg. of P.P.D. Two monkeys were not tested (4 and 55) and three monkeys (34, 35 and 36) did not react to tuberculin on several occasions at monthly intervals, although they were tuberculous at autopsy. Eight monkeys had received chemotherapeutic treatment before autopsy.

TABLE 1
DISTRIBUTION OF TUBERCULOUS LESIONS IN MONKEYS

Author	*No.*	*Lung*	*Bronchial and mediastinal lymph-nodes*	*Pleura*	*Intestine*	*Mesenteric lymph nodes*	*Liver*	*Kidney*	*Spleen*	*Peritoneum and omentum*	*Lesions in one body cavity not in other*	
											Thorax	*Abdomen*
Scott (1930)	50	50	47	34	14	19	33	28	36	27	0	2
Present investigation	20	20	20	9		0	12	7	18	7	3	0
Percentage*	70	100	96	61	20	27	64	50	77	49	4	3
Fox (1923), percentage*	204	90			15		66	37	79			

* Fox's percentage figures have not been combined with those of the first two investigators, as they were incomplete.

The exact time when the monkeys were infected is not known, but if one assumes that they react to tuberculin four weeks after infection monkeys 25 and 27 were seriously ill ten weeks after infection and monkey 26 was extensively diseased 32 weeks after infection. The clinical histories and post-mortem findings are summarised in the protocols and in Tables 1 and 2, and the clinical history of monkey 35 is given in greater detail.

TABLE 2

THE EFFECT OF STREPTOMYCIN AND STREPTOMYCIN PLUS DAPSONE ON NATURAL TUBERCULOSIS IN MONKEYS

Treatment	*Monkey No.*	*Time to death in wks.*[c]	*Macroscopic pathological assessment (d)*			*Change in Wt. during experiment kg.*	*Sedimentation rates (m.m.)*				*Average*
			Lungs	*Bronchial lymph-nodes*	*Total score incl. lesions in "other organs"*		*4th wk.*	*7th wk.*	*9th wk.*	*20th wk.*	
Streptomycin 10 mg. once daily	28[b]	24 K	2·0	0·5	2·5	+0·1	6·0	9·0	6·0	2·0	5·8
	29[a]	24 K	2·5	5·0	12·0	+0·2	17·0	7·0	2·0	2·0	7·0
	30[a]	24 K	5·0	4·5	12·0	+0·4	5·0	10·0	2·0	2·0	4·8
	Average		3·2	3·3	8·8	+0·2	9·3	8·7	3·3	2·0	5·8
Streptomycin 10 mg. once daily + *sulphone* 10 mg. twice daily	31[b]	24 K	2·5	2·0	4·5	+0·2	8·0	4·0	3·0	2·0	4·3
	32[b]	24 K	1·5	0·5	3·5	+0·1	11·0	5·0	4·0	1·0	5·3
	33[b]	24 K	4·5	4·5	10·3	0	6·0	1·0	0	1·0	2·0
	Average		2·8	2·3	6·1	+0·1	8·3	3·3	2·3	1·3	3·8
Controls	25[a]	2·7 D	5·0	9·0	16·0						
	26[a]	24 K	14·0	12·0	37·0	—1·0	11·0	9·0	10·0	16·0	11·5
	27[a]	2·7 D	14·0	7·0	29·0						
	Average		11·0	9·3	27·3						

[a] Monkeys negative to tuberculin two months before commencement of experiment, but positive one month before.

[b] Monkeys positive to tuberculin two months before commencement of experiment.

[c] K indicates that the monkey was killed when well.

D " " " " *in extremis.*

[d] Sum of individual scores as explained under "Materials and Methods."

Distribution and Nature of Lesions

The distribution of lesions is shown in Table 1. The lungs and bronchial lymph nodes of all monkeys were affected, but the disease was confined to the thorax in only three. The mesenteric lymph nodes were not affected and the findings left little doubt that the route of infection was the respiratory tract.

The lungs of monkeys 23 and 25 to 33 were x-rayed at post-mortem examination. The primary focus was obviously in the right apex in monkey 23 and in the hilum of the right diaphragmatic lobe in monkeys 33 and 32. The site or probable site of the primary focus in all monkeys is shown below:—

	Left apical lobe	*Right apical lobe*	*Right diaphragmatic*	*Left diaphragmatic*
	28	4? 36?		1, 2
	34	23 R	32, 33, 26	29? 34
	31	35, 25, 44, 3*	27, 55	30, 45, 36, 3
Total	3	7	5	8

*Cardiac

The findings in monkey 23 were of particular interest, in that there was a well marked primary complex in the right lung and bronchial lymph node and extensive bronchogenic tuberculosis in both lungs, yet the left bronchial lymph node showed no macroscopic lesions. The same tendency was observed in monkeys 25, 27,28, 30, 33 and 35, although it was not always so marked. It would seem, therefore, that as in man, secondary lesions give rise to much less reaction in the bronchial lymph nodes than does the primary lesion.

The illustrations in this paper and those given by Francis (1956) show that monkeys display all the classical features of tuberculous lesions, namely, cavitation, caseation, epithelioid cells, characteristic giant cells, macrophages, lymphocytes and some fibrosis.

Treatment of Natural Tuberculosis

The main experiment was performed on nine monkeys from a consignment that had arrived at the laboratory about three months before treatment commenced. Five of the monkeys had reacted to P.P.D. tuberculin two months before the experiment began, and four had failed to react to this test, but reacted to a test one month before the beginning of the experiment. Despite the fact that they were infected with tuberculosis the nine monkeys improved in condition during the three months between their arrival and the commencement of the experiment.

The nine monkeys were divided into three groups on the basis of their weights, sedimentation rates, and roentgenograms (Table 2). All monkeys except the controls were given 10 mg. of streptomycin per kg. subcutaneously once daily. In addition, one group was given 5 mg. of 4:4/-diaminodiphenyl-sulphone (dapsone) per kg. orally once daily, but since examination of the blood revealed no dapsone the dose was raised ten days later to 10 mg/kg. twice daily. This dose produced average morning blood concentrations of 1.28, 0.98 and 1.16 mg/100 ml. and evening blood concentrations of 0.81, 0.75 and 0.81 mg/100 ml. in the three monkeys. The results are shown in Table 2. It will be seen that the disease progressed in the untreated monkeys and two died, but the treated monkeys behaved quite differently. Those treated with streptomycin and dapsone gained 0.23 kg. and those treated with streptomycin alone 0.07 kg. The monkeys on streptomycin and dapsone appeared to improve more rapidly in clinical condition and it will be seen that in

the 7th week their average sedimentation rate was 3.3 mm., whereas that of the monkeys on streptomycin alone was still 8.7 mm. The treated monkeys and the surviving control monkey were killed 24 weeks after the commencement of the experiment. The pathological assessment of lesions is shown in Table 2 and representative autopsy findings in the protocols and figures. The total pathological score was 8.8 in the monkeys treated with streptomycin and 6.1 with streptomycin plus dapsone. The pulmonary lesions in treated monkeys were obviously not active and when portions were ground and inoculated into two guinea-pigs, and onto Loewenstein-Jensen medium, bacteria were obtained from only one monkey in each group (Table 2). Three strains of tubercle bacilli from each of these monkeys were tested for streptomycin sensitivity and they were all fully sensitive and gave growth of the human type on Loewenstein-Jensen medium.

Monkey 35. This monkey had four tuberculin tests at monthly intervals, which were all negative. When received it showed dyspnoea, cyanosis and great weakness. Roentgenographic examination showed severe consolidation of the right lung (Fig. 1). It was given 40 mg. of streptomycin per kg. subcutaneously and three days later 10 mg. of dapsone per kg. were given orally; the administration of both drugs was continued once daily. The monkey began to improve at once and five days after treatment began it was no longer cyanosed and had become quite active.

After two weeks, roentgenographic examination showed considerable clearing of the lungs and it was obvious that the right apical lobe was the one most severely affected, and that there were probably small cavities in the right diaphragmatic lobe. After 15 weeks there was further clearing in both these lobes (Fig. 2) which was complete after 24 weeks (Fig. 3). Treatment was continued for 56 weeks, when the monkey appeared normal, the weight had increased from 2.8 kg. to 4.1 kg. and the sedimentation rate had steadily decreased from 14 to 1 after seven weeks' treatment, and was 1 on six subsequent occasions up to 45 weeks' treatment after which no further tests were performed.

On the last day of treatment the blood contained 0.88 mg. of dapsone per 100 ml. before dosing (i.e. 24 hours after the previous dose), and 1.12 mg. per 100 ml. four hours after dosing. The absorption spectra of the blood of monkey 35 was compared with that of a monkey treated with streptomycin and with that in two normal monkeys: methaemoglobinaemia was not detected. The monkey had a red cell count of 5 million, a white cell count of 14,000 and a haemoglobin concentration of 12.9 g/100 ml. A culture was recovered from a tracheal swab after 16 weeks' treatment, and was fully sensitive to streptomycin. It gave a smooth growth on Loewenstein-Jensen medium, and growth on Dorset's egg medium was not improved by glycerine. It was, therfore, probably of the bovine type, but unfortunately no animal tests were performed.

The monkey was very active for all but the first few weeks of the 56 weeks of treatment and remained well for about 12 weeks after treatment was stopped, but after 17 weeks it was dull and listless and the weight had decreased from 4.1 to 3.2 kg., although no very distinct lesions could be seen on roentgenographic examination. Autopsy showed that the

right apical lobe of the lung was collapsed and red, it contained a few hard millet-seed lesions as did the rest of the lung tissue. The right cephalic bronchial lymph node was moderately enlarged and caseous (T.B.3), but the other nodes were only slightly enlarged. The spleen was much enlarged and attached to the stomach and kidney; it contained large caseous areas (T.B.1). There were smaller lesions in the liver, kidney and omentum.

Treatment of Experimental Tuberculosis

Toxicity tests with 7438 (Francis, *et al.*, 1954) showed that one monkey tolerated doses of 30 mg/kg. twice daily for 60 days without showing any adverse haematological changes, and during this period it increased in weight from 3.2 to 3.6 kg. Average blood concentrations of 3.5 to 5.5 mg/100 ml. of 7438 were obtained.

Three monkeys were inoculated intranasally with 0.1 mg. of human tubercle bacilli into both the right and left lungs. The monkeys began to cough 9 to 11 days after infection. They were roentgenographed on the 13th day under nembutal anaesthesia. Unfortunately monkey 6, which was weak, died and post-mortem examination revealed extensive consolidation of both lungs and small lesions in the liver and spleen. The roentgenograms indicated that monkeys 5 and 7 had similar pulmonary lesions. On the 14th day both monkeys were coughing, but monkey 5 was fairly bright and eating, whereas monkey 7 was dull, coughing and not eating. It was given 20 mg. of streptomycin per kg. twice daily, and monkey 5, 30 mg. of 7438 per kg. twice daily by mouth. The effect was striking, and by the 16th day monkey 7 was active, had stopped coughing and was eating well, whereas monkey 5 was now dull, coughing badly, but still eating fairly well. It died on the 28th day with extensive tuberculous bronchopneumonia. The treatment of monkey 7 with streptomycin continued for 11 weeks and the monkey survived for a further 23 weeks, when it was killed because of dyspnoea. Autopsy showed that the lesions in the lungs had undergone much resolution but the bronchial lymph nodes were greatly enlarged and obviously the cause of the dyspnoea.

The subsequent experiment, except for the results with 7438, was described by Francis, Spinks and Stewart (1950). The monkeys were infected with 0.01 mg. of tubercle bacilli into the right and left lungs and all were showing symptoms of malaise or anorexia 14 days later when treatment was begun with streptomycin, *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (8388), or 100 mg. of 7438 per kg. twice daily. The controls died with extensive lesions of tuberculosis 22 to 33 days later. The large dose of 7438 was chosen, as 30 mg. per kg. had produced little effect in the previous experiment, but it proved to be toxic and monkey 19 died 17 days after infection, i.e. 3 days after treatment began, and monkey 17, 30 days after infection. Both had extensive lesions of tuberculosis. The other monkey treated with 7438 survived for 86 days, as did all the

monkeys treated with 500 mg. of 8388 per kg. or 40 mg. of streptomycin per kg. daily. They were then killed and post-mortem examinations performed. The detailed results, except with 7438, were recorded (Francis, *et al.*, 1950), and typical lesions illustrated. The average pathological score with streptomycin was 15.7, with 8388, 27.3, and with the one surviving monkey treated with 7438, 20. It is evident that 7438 was much less effective than streptomycin and probably less effective than 8388, as two monkeys treated with 7438 had died before the completion of the experiment with extensive lesions of tuberculosis.

Immunization of Monkeys with B.C.G.

The foregoing pathological observations, especially those in monkey 35, indicated that the monkey would develop immunity to tuberculosis, but the results on immunization of monkeys with B.C.G. are somewhat conflicting (Irvine, 1949). Some workers, particularly those in the tropics, have reported very good results, whereas others, including Griffith (1931), only poor results even with large doses of B.C.G.: one gains the impression, however, that Griffith's experiments on monkeys were not carried out under ideal conditions.

Monkey 24 was vaccinated subcutaneously with 1 mg. of a laboratory strain of B.C.G. which had been obtained from the Veterinary Laboratory, Weybridge. It did not react to 0.01 mg. P.P.D. 3 weeks later, but did react 6 weeks after vaccination. It was infected intranasally into each lung with 0.01 mg. of human tubercle bacilli 7 weeks after vaccination. The monkey was killed when ill 13 weeks after infection, and had lesions of generalised tuberculosis. Unfortunately no control monkey was available, but other monkeys infected with this dose of tubercle bacilli have died in about 4 weeks (Francis, *et al.*, 1950).

Four monkeys were inoculated intradermally with 0.01 ml. of the standard B.C.G. vaccine containing 0.75 mg./ml. of bacilli used for intradermal vaccination of man. Nine weeks later the vaccinated monkeys reacted to the intrapalpebral injection of 0.01 mg. of P.P.D. tuberculin and they, together with four control monkeys that had not reacted to tuberculin, were infected intranasally with 10^{-4} mg. of human tubercle bacilli. Ten weeks later all the monkeys had died or were killed *in extremis,* and there was no difference between the lesions of generalised tuberculosis seen in the vaccinated and control groups.

In a subsequent experiment 1 ml. of standard B.C.G. vaccine was given subcutaneously. Ten weeks later the vaccinated animals, but not two control monkeys, reacted to the intrapalpebral injection of 0.01 mg. of P.P.D. tuberculin, and all were infected intranasally with 5×10^{-4} mg. of human tubercle bacilli. The two control monkeys died with generalised tuberculosis 13 and 17 weeks later. Unfortunately both vaccinated animals had to be killed 30 weeks

after infection. Autopsy revealed a number of small lesions in the lungs and caseous bronchial lymph nodes, the spleens contained a few caseous foci and numerous small foci, there were a few tuberculous foci in the cortices of the kidneys; the livers were macroscopically normal. In this experiment with the larger dose of B.C.G. there was an obvious immunizing effect and the vaccinated animals would probably have lived three times as long as the unvaccinated.

DISCUSSION

Tuberculosis in monkeys is usually an acute process, but the fact that secondary lesions sometimes fail to produce macroscopic changes in the regional lymph nodes, and that a classical histological picture is produced with some fibrosis, indicates that tuberculosis in the monkey more closely resembles that in primitive man than does tuberculosis in other animals. These observations also indicate that it would be possible, if breeding experiments could be performed, to select resistant monkeys in which the disease would resemble tuberculosis in urban man much more closely than it does in resistant animals such as the dog, mouse, or even the bovine (Francis, 1947; Francis, 1956).

It was observed that bacilli were most numerous in necrotic tissue and less numerous in caseous tissue, and in apparently healthy cells. This is in accord with the findings of Canetti (1946) in man, and the meticulous and extensive observations of Eastwood (1911) in a variety of species. As Canetti says, necrotic tissue, particularly that lining a cavity, should be regarded as a foreign body: bacilli find it a favourable medium for multiplication which is apparently beyond the reach of the immune mechanisms of the body. It is emphasised by Francis (1956) that although bacilli may be numerous in necrotic tissue in man, monkeys and various other animals that become allergic, they are seldom numerous within healthy cells except when allergy declines before death: and one may add in the early stages of primary infection (Canetti, 1946; Rich, 1951) presumably before allergy has developed.

The results of B.C.G. vaccination provide further support for the view (Francis, 1949) that immunization would reduce the severity of primary tuberculous infection in man, and indicate that immunization might reduce the spread of tuberculosis in monkey colonies. It is interesting that with an insufficient dose allergy, but not immunity, was produced. Three of 18 tuberculous monkeys failed to react to 0.01 mg. of P.P.D. so it would appear that a larger dose should be used when testing monkeys.

It is interesting that although the rabbit and guinea-pig appear to be almost as susceptible as the monkey to experimental tuberculosis (Francis, 1956), spontaneous tuberculosis in these species is a pathological curiosity, whereas it may be the chief cause of death in monkey colonies.

Streptomycin appeared to have as striking an effect in reversing a severe tuberculous process (Figs. 1 to 3) in the monkey, as it does in the guinea-pig (Feldman, 1946). Cultural and biological examination of lesions in monkeys that had had a moderate degree of tuberculosis when treatment began indicated that the lesions were sterilised. However, two severely diseased monkeys (7 and 35) that made a striking clinical recovery, eventually relapsed after treatment was withdrawn. The results in monkey 7 indicate that streptomycin has a greater effect on pulmonary than on glandular tuberculosis, as it does in man (Bentley, Grzybowski and Benjamin, 1954; Gale, 1954; Williams and Anderson, 1953), and the results in monkey 35 indicates that streptomycin has a greater effect on pulmonary than on extrapulmonary tuberculosis.

Dapsone produced an additive effect when given with stretpomycin, as it does in the tuberculous guinea-pig but not in the dog (Francis, 1953). It appeared to be well tolerated by monkeys, but its toxicity for various species varies considerably (Stewart Lawrence and Francis, 1953); thus it is less toxic than sulphanilamide for cattle and produces no methaemoglobinaemia, cyanosis or nervous symptoms.

CONCLUSIONS

Information on tuberculosis in monkeys is briefly reviewed and the autopsy findings on 20 naturally infected monkeys are recorded. Monkeys showed all the classical features of tuberculous lesions—cavitation, caseation, epithelioid and characteristic giant cells, macrophages and lymphocytes and often, even in quite acute processes, some fibrosis. As in man there was a tendency for secondary lesions to produce only slight changes in the regional lymph nodes.

Three of 17 monkeys did not react to tuberculin on several occasions at monthly intervals, although they were tuberculous at autopsy.

A small dose of B.C.G. produced allergy but not immunity, a larger dose produced both allergy and immunity.

Treatment of naturally infected monkeys with streptomycin greatly retarded the development of tuberculosis and appeared to sterilise the lesions, but two monkeys which were serioulsy ill when treatment began and made a remarkable recovery, eventually relapsed after treatment was withdrawn.

Dapsone (4:4/-diaminodiphenylsulphone) appeared to increase slightly the action of streptomycin. Methaemoglobinaemia was not detected in a monkey that had received dapsone for 56 weeks, and had blood concentrations of over 1 mg/100 ml.

The therapeutic margin with 5-amino-7-methyl-1:2:4:6-tetraazaindene (7438) was small and the drug was a much less satisfactory therapeutic agent in monkeys than streptomycin.

ACKNOWLEDGMENTS

I am indebted to Mr. W. Russell for technical assistance, and to Mr. A. B. Halam and Mr. E. Young, of Imperial Chemical Industries, and Mr. E. W. Hollywood, of the University of Queensland, for the photographs.

REFERENCES

Bentley, F. J., Grzybowski, S., and Benjamin, B. (1954). *Tuberculosis in Childhood and Adolescence.* The National Association for the Prevention of Tuberculosis; London.

Canetti, G. (1946). *Le Bacille de Koch dans la Lésion Tuberculeuse du Poumon;* Paris.

Cobbett, L., and Griffiths, A. S. (1913). *Roy. Com. Tuberc.,* Fin. Rep., App. II, Suppl. Vol.

Dubos, R., and Dubos, J. (1953). *The White Plague: Tuberculosis, Man and Society.* Victor Gollancz Ltd.; London.

Eastwood, A. (1911). *Roy. Com. Tuberc.,* Fin. Rep. Pt. II, App. Vol. V.

Edwards, P. W., Penman, A. C., and Cutbill, L. J. (1952). *Brit. med. J., i,* 1224.

Feldman, W. H. (1946). *J. roy. Inst. publ. Hlth,* **9**, 343.

Fox, H. (1923). *Disease in Captive Wild Mammals and Birds,* J. B. Lippincott Co.; Philadelphia.

Francis, J. (1947). *Bovine Tuberculosis.* Staples Press Ltd.; London; (1949). *Lancet, ii,* 549; (1953). *Brit. J. Pharmacol.,* **8**, 259.

Francis, J. (1956). *Tuberculosis in Animals and Man: a Study in Comparative Pathology.* Cassells & Co. Ltd.; London.

Francis, J., and Hoggarth, E. (1953). *Brit. J. Pharmacol.,* **8**, 125.

Francis, J., Martin, A. R., Rose, F. L., and Spinks, A. (1954). *Ibid.,* **9**, 437.

Francis, J., and Spinks, A. (1950). *Ibid.,* **5**, 565.

Francis, J., Spinks, A., and Stewart, G. T. (1950). *Ibid.;* **5**, 549.

Gale, G. L. (1954). *Amer. Rev. Tuberc.,* **70**, 610.

Griffiths, A. S. (1931). *Spec. Rep. Ser. Med. Res. Counc., London,* No. 152. H.M. Sta. Off., London.

Habel, K. (1947). *Amer. Rev. Tuberc.,* **55**, 77.

Irvine, K. Neville (1949). *B.C.G. Vaccination in Theory and Practice.* Blackwell Scientific Publications; Oxford.

Kennard, M. A., Schroeder, C. R., Trask, J. D., and Paul, J. R. (1939). *Science,* **89**, 442.

Lowe, J. (1950) *Lancet, i,* 145.

Rich, A. R. (1951). *The Pathogenesis of Tuberculosis.* Blackwell Scientific Publications; Oxford.

Schroeder, C. R. (1938). *Amer. J. publ. Hlth.* **28**, 469.

Schroeder, C. R. (1938). *Zoologica N.Y.,* **23**, 397.

Scott, H. H. (1930). *Spec. Rep. Ser. Med. Res. Counc., London,* No. 149 H.M. Sta. Off.; London.

Skavlem, J. H. (1953). *Trans. 12th Conf. Chemoth. of Tuberc. Veterans Admin. U.S. Army & Navy,* p. 143.

Stewart Lawrence, J., and Francis, J. (1953). *The Sulphonamides and Antibiotics in Man and Animals.* H. K. Lewis & Co. ; London.

Straus, I. (1895). *La Tuberculose et son Bacille.* Paris.

Williams, H., and Anderson, C. (1953). *Qt. J. Med.,* New Series **22**, 295.

APPENDIX

MONKEY 26. A control monkey in the main chemotherapeutic experiment. It had reacted to tuberculin test one month, but not two months, before the experiment began. It was killed at the end of the experiment when not obviously ill, i.e. about 32 weeks after becoming infected (Table 2).

The *right lung* was adherent ot the diaphragm. There was a large cavity containing caseous pus in the *right diaphragmatic lobe*, the adjoining tissue contained very numerous epithelioid cells and giant cells of varying shape, some with groups of central nuclei (as in Fig. 8). There were small lesions in other parts of the *right* and *left lungs*. The *right bronchial lymph nodes* were much enlarged and contained soft caseous pus. There were numerous epithelioid cells and a moderate number of giant cells (Fig. 4). The *left bronchial lymph nodes* were moderately enlarged and caseous with two calcified points (T.B. 2–3).

There were fairly severe tuberculous lesions in the *omentum*, *liver* and *spleen*, and a few foci in one *kidney*.

MONKEY 27. A control monkey in the main chemotherapeutic experiment (Table 2); it reacted to the tuberculin test one month, but not two months, before the experiment began. It became ill and showed dyspnoea 28 days after the experiment began, and died 2 days later, i.e. about 10 weeks after becoming infected.

There was acute cavitating bronchopneumonia in the *apical lobe* of the *right lung* (Fig. 5). The histological picture is shown in Fig. 6. The *cardiac* and *diaphragmatic lobes* contained similar but less severe lesions. The *left lung* contained several small caseous lesions. There were a few tubercles on the *omentum* and *peritoneum*, and in the *liver* (Fig. 7), *spleen* and *kidney*.

MONKEY 29. This monkey reacted to tuberculin test one month, but not two months, before the experiment began. It was treated with streptomycin for 24 weeks before autopsy (Table 2).

The *left lung* contained two small cavitated foci (Fig. 8) with considerable surrounding fibrosis. The *left bronchial lymph node* was much enlarged and cavitated. The *right lung* and *bronchial lymph node* were normal. There were several small lesions in the *omentum*, *spleen*, *kidney* and *liver* (Fig. 9).

MONKEY 30. This monkey reacted to tuberculin test one month, but not two months, before the experiment began. It was treated with streptomycin for 24 weeks before autopsy (Table 2).

The *lungs* contained a few small scattered foci, some of which contained the bodies of pneumonyssus parasites. Other lesions were tuberculous (Fig. 10). The *right bronchial lymph node* was enlarged, cavitated and contained caseous pus; epithelioid and numerous giant cells. The *left bronchial lymph node* was macroscopically normal, but contained numerous epithelioid cells and yellow translucent debris, and eosinophil cells, indicative of parasitic infection.

The *liver* contained a few subcapsular tuberculous foci. The omentum was adherent to the intestines and contained numerous parasitic nodules, which could have been confused with tuberculous lesions.

MONKEY 33. It reacted to tuberculin two months before the experiment began. It was treated with streptomycin and dapsone for 24 weeks before autopsy (Table 2).

The *pleura* was slightly adherent to the chest wall. The *diaphragmatic lobe* of the *right lung* contained a small caseo-calcareous focus (Fig. 11) surrounded by some fibrous tissue. The *left apical lobe* contained a small gritty focus with the histological appearance of a parasitic infection. The *right bronchial lymph nodes* were converted to caseo-calcareous masses (T.B.1), surrounded by fibrous tissue. They contained some yellow debris indicative of parasitic infection.

There were small tuberculous lesions in the *liver* and *spleen*.

[*Received for publication, September 17th* 1955]

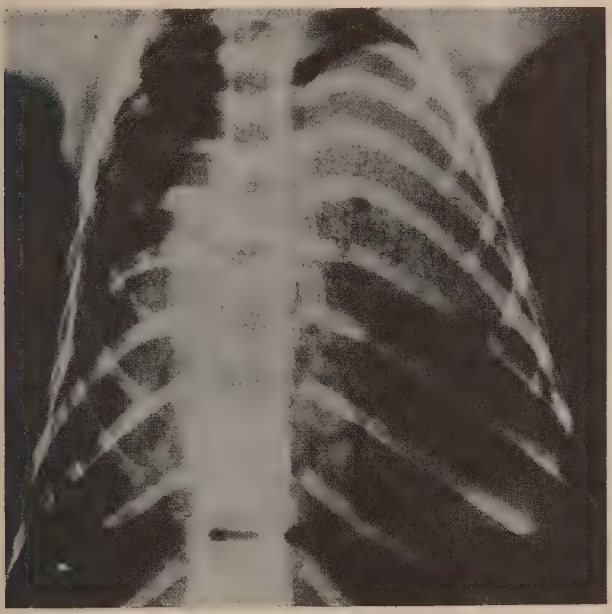
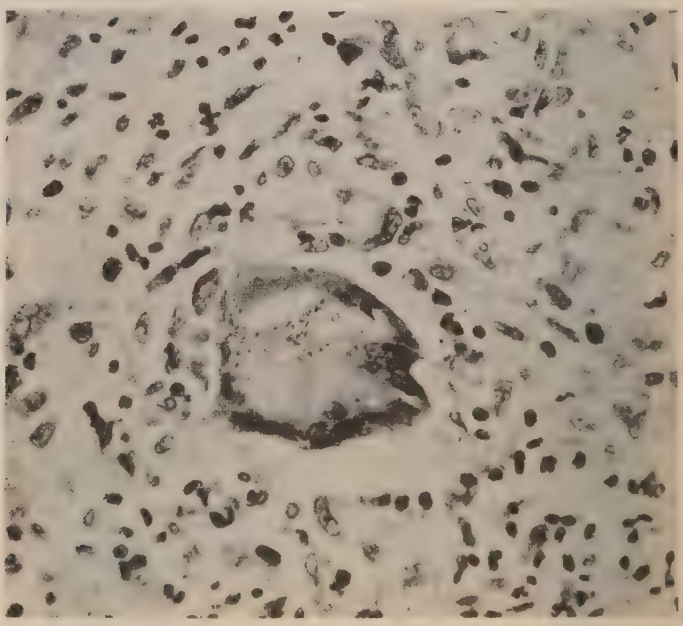


FIG. 1

FIG. 2

FIG. 3



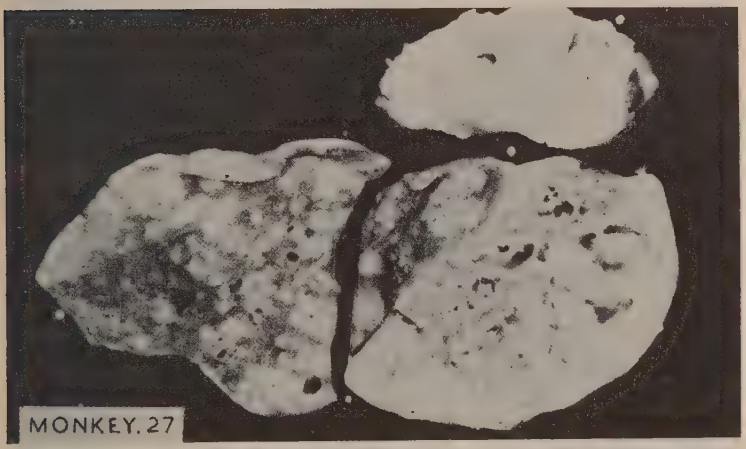


FIG. 5

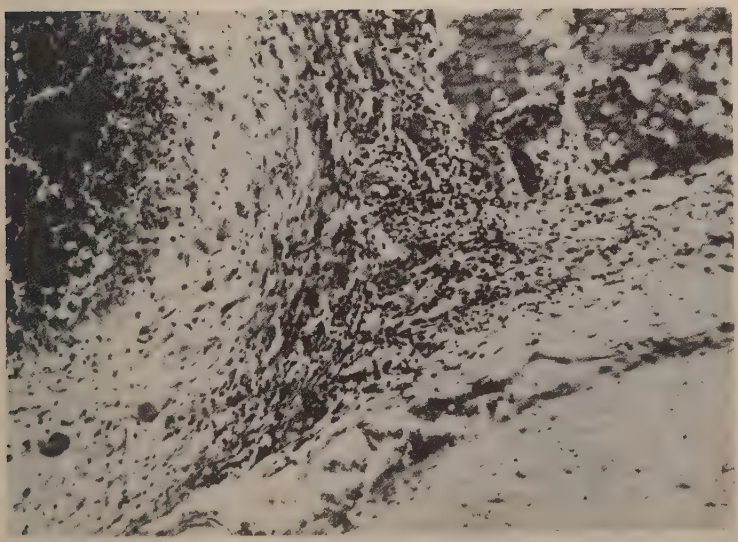


FIG. 6

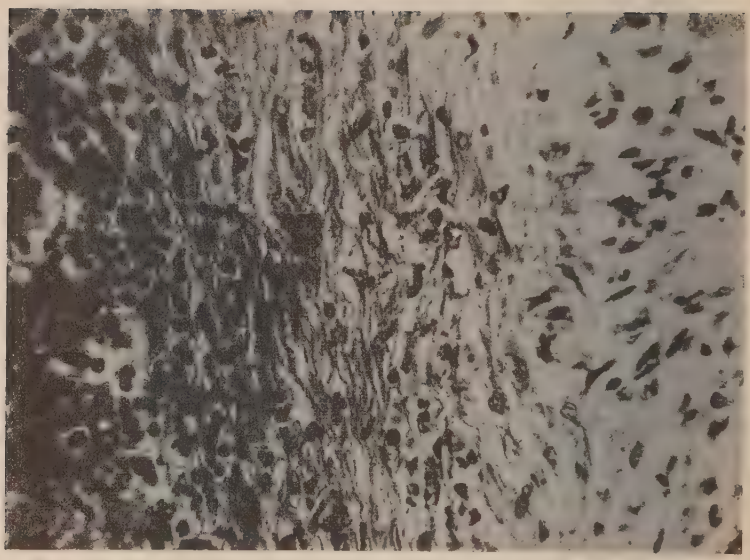


FIG. 7

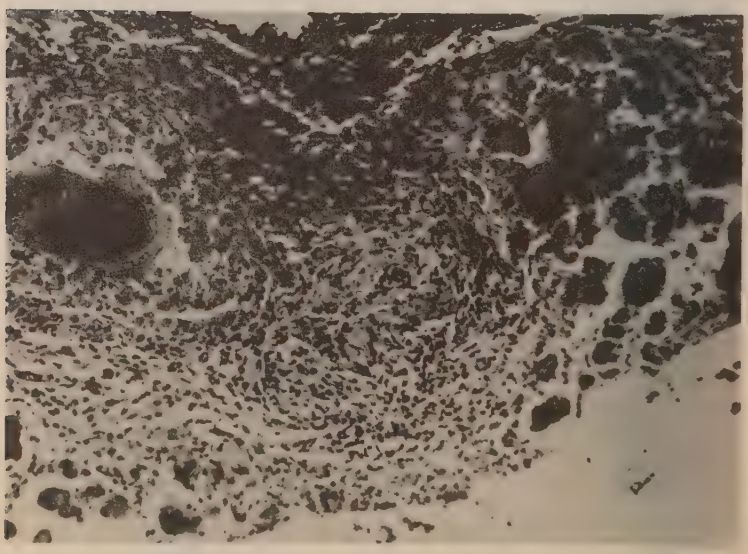


FIG. 8

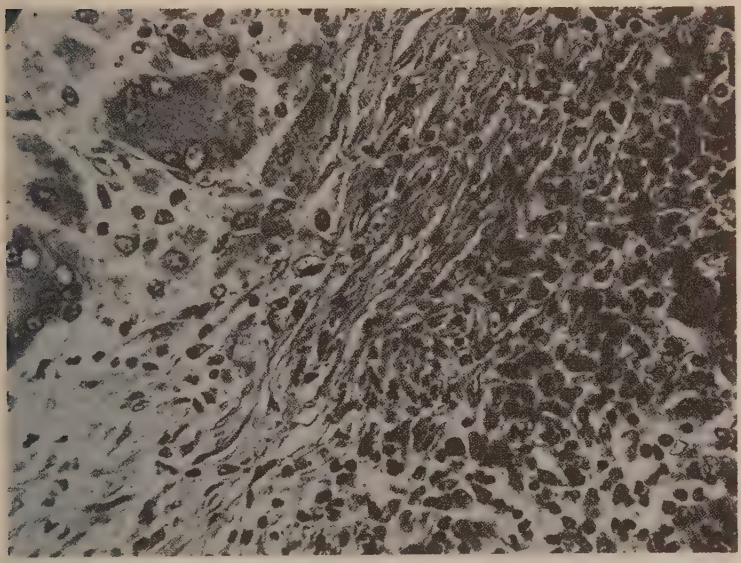


FIG. 9

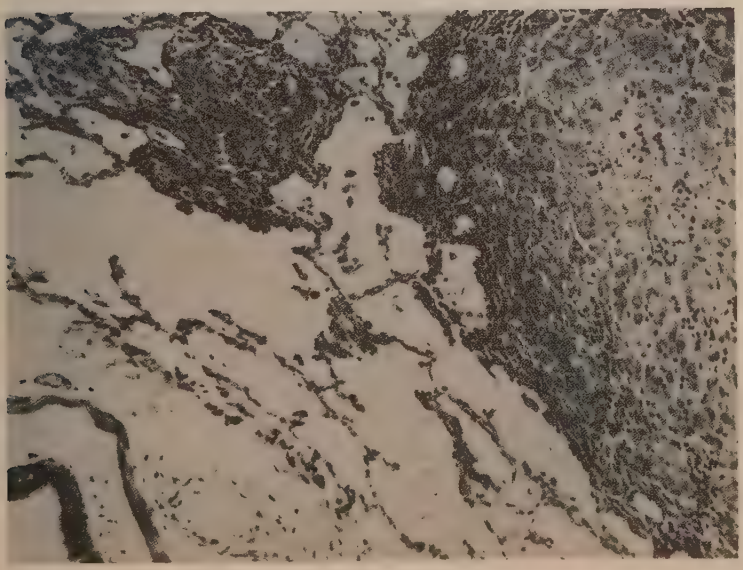


FIG. 10

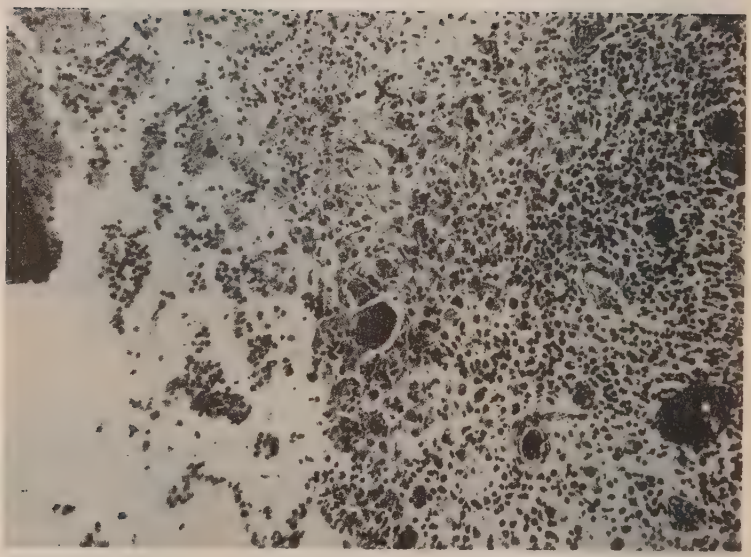


FIG. 11

LEGENDS TO FIGURES*

Fig. 1 Chest roentgenogram of monkey 35 at the time treatment commenced showing marked consolidation of the right apical lobe and a small central cavity. There is consolidation of the caudal portion of the right diaphragmatic lobe and probably some cavitation.

Fig. 2. Chest roentgenogram of monkey 35 after 15 weeks treatment with streptomycin and dapsone. There is considerable clearing of the right apical lobe, but diffuse consolidation and small cavities can be seen in the diaphragmatic lobe.

Fig. 3. Chest roentgenogram of monkey 35 after 24 weeks treatment. There is complete clearing of the right lung.

Fig. 4. The right bronchial lymph node of monkey 26. A giant cell in the centre contains tubercle bacilli and is surrounded by epithelioid and other inflammatory cells. ZN × 510

Fig. 5. Right lung of monkey 27. Acute cavitating bronchopneumonia in the apical lobe and less severe lesions in the diaphragmatic lobe. The caseous and cavitated bronchial lymph-node is shown above. × 1

Fig. 6. Lung of monkey 27. A necrotic area on the left is surrounded by a zone of slight fibrosis. There is exudate, and breakdown of alveolar walls between this zone and the fibrous pleura above. H & E × 85

Fig. 7. Liver of monkey 27. The margin of a necrotic zone can be seen on the right, and a zone of fibrous tissue with lymphocytes and liver cells on the left. H & E × 400

Fig. 8. The diaphragmatic lobe of the left lung of monkey 29. There are several giant cells in the cavity wall, and one contains numerous centrally placed nuclei. There was considerable surrounding fibrosis and adjoining areas of bronchopneumonia. H & E × 200

Fig. 9. Liver of monkey 29. Liver cells can be seen on the left, then a zone of fibrosis, and giant cells. There was a necrotic area to the right of the giant cells. H & E × 510

Fig. 10. Lung of monkey 30. There is a portion of a bronchiole in the lower left-hand corner. The centre of the larger epithelioid focus is slightly necrotic (T.B. 1), but there is little inflammatory exudate in the alveoli. H & E × 200

Fig. 11. Lung of monkey 33. The wall of a cavity with caseous and calcified tissue on the left. On the right epithelioid and giant cells can be seen and there are several blood vessels. H & E × 200

*The numbers following the letters "T.B." indicate the number of tubercle bacilli as defined under "Materials and Methods". Further details concerning the monkeys are given in the protocols.

DUAL VACCINATION OF DOGS AGAINST THE CANINE DISTEMPER COMPLEX AND CANINE VIRUS HEPATITIS

By

W. Mansi

Veterinary Laboratory, Ministry of Agriculture, Weybridge.

INTRODUCTION

During the last few years it has become obvious that the clinical picture of canine distemper in dogs can be very variable, and it is often impossible to give a sound diagnosis on clinical grounds only. The difference in the clinical picture is probably due to variation in the affinities of different strains for different tissues (Mansi, 1951) and partly to concurrent infections with canine virus hepatitis (Flint, 1953). Gillespie, Robinson and Baker (1952) reported that a dual infection with both viruses results in a high mortality rate and also showed that recovered animals were immune to both infections. In a previous communication (Mansi, 1953), the results of the conjunctival instillation of hepatitis virus in dogs were reported. Dogs treated in this manner showed a thermal reaction, conjunctivitis, oedema of the conjunctival sac (eye reaction), tonsillitis and lymphadenitis, followed by recovery, about 9 to 12 days after instillation of the virus, and immunity to further infection. Haig (1948) first reported the successful propagation and subsequent attenuation of the canine distemper virus on the chorio-allantoic membrane of the developing chick embryo; since then, avianised-virus has been used in different parts of the world and its value to protect dogs against experimental and natural infection is now well established.

In this present work, it was decided to explore the possibility of a dual vaccination against canine distemper virus (DCX) and canine hepatitis virus (HCC) virus infections by combining the two methods.

MATERIALS AND METHODS

Dogs. Ninety-nine dogs were obtained from litters born from bitches on the premises which had been immunised against DCX by the subcutaneous inoculation of avianized virus-vaccine and against HCC mainly by the conjunctival instillation method. Forty-seven dogs were also purchased from unknown sources when they were 4 to 6 weeks old. All dogs of either group on reaching the age of six weeks were isolated and their blood was tested for the presence of complement-fixing antibodies of both virus infections; none was found to be positive.

Dogs were usually vaccinated at the age of 3 months. Nineteen dogs which had been previously vaccinated against one virus infection at a time were employed as known immune controls. Fifty-six dogs belonging to members of the staff were also vaccinated, primarily as a prophylactic measure. Of these, 10 were survivors of an outbreak of dual infection with both viruses in which the mortality was 52·6 per cent. Forty-two of these dogs were 3 to 6 months old and the remaining fourteen were between 2 and 8 years of age.

A total number of 221 dogs was thus employed in this work.

Avianised canine distemper virus (DCX). The following strains were used: Onderstepoort, Green (locally adapted), Glasgow S123, (locally adapted). There was no immunological difference between the three strains. The potency of the virus was estimated by a ferret-protection test. A dilution of 1/100 of infected chorio-allantoic membrane in beef-heart infusion broth at pH 7·4, prepared and freeze-dried at once, was used. The minimum immunising dose for a ferret varied from 1×10^{-5} to 1×10^{-6} ml. The distemper virus-vaccine was inoculated subcutaneously in 134 dogs and instilled separately or mixed with HCC virus in 25 dogs. The dose was not less than 1000 minimum ferret immunising doses.

Canine virus hepatitis (HCC). A virulent strain ("Bec") was used for immunization and was introduced by conjunctival instillation. One drop of a dilution of 1/100 of infected dog liver suspension in broth was usually employed.

Vaccination. All virus-vaccines were freeze-dried. In the later part of the work the two vaccines (DCX and HCC) were pooled and then freeze-dried. One drop of the resuspended virus, prepared immediately before use, was dropped in the conjunctival sac of one eye from an ordinary eye dropper which had a very blunt end to avoid any possible risk of injury during instillation, the eye-lid was rubbed gently over the eye-ball and the excess fluid was wiped off with absorbent cotton wool. The freeze-dried virus-vaccines were found to be viable for at least three years if kept at 4°C.

Challenge tests. Challenge by inoculation was usually carried out by intravenous inoculation of a mixture of virulent canine distemper virus (100,000 M.L.D. "ferret") and canine hepatitis virus (50,000 M.I.D. "dog") in a total volume of 2 ml. This inoculum proved to be sufficient to cause serious, if not fatal, results in susceptible control animals. In addition, 40 dogs belonging to members of the staff, which had been vaccinated primarily as a prophylactic measure, have since been exposed to the normal chances of natural infection.

Owing to the shortage of accommodation, only 6 vaccinated dogs could be kept for longer than a year before challenge. Challenge was usually carried out after 7 to 15 days.

Serological confirmation. The complement-fixation test, as described in a previous communication, was used (Mansi, 1955). Sera collected before and after vaccination, and subsequently after challenge, were tested for the presence of complement-fixing antibodies of both virus groups. Tissues collected from control animals, which died of either or both infections, were tested for the presence of specific soluble antigens of both viruses. The test was very useful in detecting inapparent infections.

RESULTS

The Effect of Vaccination with HCC on Dogs already Exposed to HCC Infection

Seven susceptible and one immune puppy were placed in contact with a dog reacting to HCC. At different intervals six received the HCC virus into the conjunctival sac (Table 1). The puppy that was vaccinated at the time of exposure and that treated $4\frac{1}{2}$ hours later reacted normally to vaccination with the characteristic eye reaction. The four dogs vaccinated 24 or more hours after exposure did not show the specific eye reaction. Instead they showed symptoms of a

systemic infection, but the reaction was not severe and all four recovered. One dog, which did not receive the HCC vaccine, showed signs of infection but also recovered. The immune control showed neither local nor systemic infection and remained unaffected.

TABLE 1

No. of dogs	*Period between exposure and instillation of the virus in hours*	*Results*						
		T°	*C*	*ER*	*T*	*L*	*NF*	*Termination*
1	0	+	+	+	+	+	—	R. 10th day
1	4½	+	+	+	+	+	—	R. 10th day
1	24	+	+	—	+	+	—	R. 8th day
1	48	+	+	—	+	+	—	R. 8th day
1	72	+	+	—	+	+	—	R. 11th day
1	96	+	+	—	+	+	—	R. 7th day
1	no instillation	+	+	—	+	+	+	R. 10th day
1	immune	—	—	—	—	—	—	immune

T°: Thermal reaction *C:* conjunctivitis *ER:* eye reaction
T: tonsillitis *L:* lymphadenitis *NF:* not feeding
R: recovered

Determination of the Minimum Period Required after Dual Vaccination to Acquire Protection, and of the Duration of Immunity

Observations recorded in Table 2 show that the animals vaccinated subcutaneously with the DCX avianised virus developed immunity in three days while those immunised by the instillation method could only resist the challenge from the seventh day onwards. When challenged before the minimum period necessary for conferring immunity had elapsed, six dogs (two from the first and four from the second group) developed a form of the distemper syndrome—visceral and nervous—which proved fatal (Table 2). Animals which were challenged 3 days after DCX vaccination by the subcutaneous route or 6 days after vaccination by the instillation method were solidly immune.

Resistance to HCC developed in three days. It was found that when the eye reaction developed in one eye, the other eye if treated in the same way more than 24 hours later did not show any reaction. However, if the virus was instilled in each eye at the same time, both of them reacted. There was no interference between the mechanism as a result of immunisation with the two viruses. The eye reaction was clearly due to the HCC virus, since DCX virus alone did not produce any such effect.

TABLE 2

RESULT OF CHALLENGING DOGS AT DIFFERENT PERIODS AFTER DUAL VACCINATION

No. of dogs	*Route of administration of*		*Period between vaccination and challenge in days*	*Result of challenge by intravenous inoculation of virulent DCX and HCC viruses*
	HCC vaccine	*DCX avianised vaccine*		
1	Inst.	S/C	0*	Died 13th day, showed symptoms of DCX.
1	,,	S/C	1*	Showed symptoms of systemic infection of DCX & HCC; recovered
1	,,	S/C	2	Showed symptoms of systemic infection of DCX & HCC; died 18th day
1	,,	S/C	3	Unaffected—immune
4	,,	Inst.	4	All four showed symptoms of DCX infection, all died; 10th, 10th, 14th and 18th days
1	,,	S/C	4	Unaffected—immune
8	,,	S/C	5	,, ,,
4	,,	S/C	7	,, ,,
8	,,	Inst.	8	,, ,,
16	,,	S/C	8	,, ,,
18	,,	S/C	10	,, ,,
9	,,	Inst.	11	,, ,,
2	,,	S/C	13	,, ,,
17	,,	S/C	15	,, ,,
4	,,	S/C	59	,, ,,
2	,,	S/C	168	,, ,,
6	,,	S/C	365	,, ,,
24	Non-vaccinated dogs used as controls in the immunity tests of vaccinated dogs above.			11 died of HCC infection. 4 died of DCX infection. 1 died of dual infection. 6 recovered after serious HCC infection 2 recovered after serious DCX infection

S/C: subcutaneous
Inst: instillation

* animals vaccinated at these periods did not show the eye reaction of HCC virus-vaccine.

Owing to the shortage of isolation accommodation, it was only possible to hold six dogs for a year, but a solid immunity was still present after this period.

FIG. 1

Dog on the left showing characteristic eye reaction.
Dog on the right showing early signs of the reaction.

Vaccination of Dogs belonging to Members of the Staff.

In all 56 dogs were vaccinated; Group A consisted of 46 dogs which had no history of illness and Group B consisted of 10 dogs, known survivors of an outbreak of DCX and HCC. From the results recorded in Table 3 it can be seen that 39 (87 per cent) dogs from Group A, average age 3 to 6 months, showed a positive reaction. These animals recovered without showing any serious illness or giving any cause for anxiety. One dog from this group which showed no eye reaction but developed a systemic infection and recovered, might have been in the incubative stage of HCC. The remaining 5 young dogs of Group A showed no eye reaction and remained otherwise unaffected; presumably they were immune at the time of vaccination. Five of the 40 reactors were revaccinated but remained unaffected. All dogs in Group B remained unaffected; presumably because they were already immune.

The first dog was vaccinated in November 1953, since when, although presumably exposed to street infections, no breakdown in immunity has been reported in the group.

TABLE 3

SUMMARY OF RESULTS OF DUAL VACCINATION OF DOGS BELONGING TO MEMBERS OF THE STAFF

	Group A. 46 dogs No history of illness				*Group B. 10 dogs Known survivors of an outbreak of DCX and HCC*
Age group	3–6 months	24 months	36 months	48 months	2–8 years
Nos. vaccinated	42*	1	1	2	10
Eye reaction positive	39	0	1	0	0
Eye reaction negative	2	1	0	2	10
Eye reaction negative but developed systemic infection and recovered	1	0	0	0	0

* 4 dogs of this group received the DCX virus—vaccine by conjunctival instillation.
The remaining dogs received the DCX virus-vaccine by subcutaneous inoculation.
All 56 dogs received the HCC virus-vaccine by conjunctival instillation.

The Possibility of Contact Infection between Vaccinated Dogs and Susceptible In Contacts

This problem was studied to determine whether susceptible dogs could become infected with HCC by exposure to vaccinated animals and, if such infection occurred, to find the maximum period for which the vaccinated dogs remained dangerous. The tests recorded in Table 4 showed that there is a danger period beginning at the

TABLE 4

No. of normal dogs exposed to one or more vaccinated dogs	*Time at which susceptible dogs were exposed to vaccinated animals (contact for three weeks)*	*Clinical results*						
		T°	*C*	*ER*	*T*	*L*	*CO*	*Termination*
6	at time of vaccination	6	6	0	6	6	2	2 died
1	4 days	1	1	0	1	1	0	Recovered
6	6 days	6	6	0	6	6	0	Recovered
17	15–44 days	0	0	0	0	0	0	Proved susceptible on challenge
1	102 days	0	0	0	0	0	0	

T°: thermal reaction *C:* conjunctivitis *ER:* eye reaction
T: tonsillitis *L:* lymphadenitis *CO:* corneal opacity
1, 2 etc.: number of dogs which reacted in each group

time the animal is vaccinated and lasting until the reaction subsides. The time never exceeded the 15 days during which previous experiments had shown virus to be recoverable and HCC complement-fixing antigen to be demonstrable in the tissues of dogs killed after infection with HCC virus (Mansi, 1955). Between 15 and 102 days after vaccination 18 dogs were exposed and remained in contact with the recovered dogs. They showed no signs of clinical infection and their blood-serum developed no complement-fixing antibodies of HCC. These dogs were susceptible on challenge (Table 4).

DISCUSSION

It is generally agreed that a living virus attenuated by passage in an unusual host, e.g. egg-adapted distemper virus, gives better protection against experimental or natural infection than a killed or inactivated virus-vaccine. With the former, protection is acquired in a comparatively short time (3 to 7 days) and one dose is often sufficient to protect for life. With killed vaccines the period required to confer immunity is longer (2 to 3 weeks), resulting in the increased risk of exposure before immunity develops. It is also often necessary to administer more than one dose.

The results of the conjunctival instillation of HCC virus, which have been reported in a previous communication, were so encoura-

ging that further investigations were carried out. Fully virulent HCC virus was used because so far it has not been possible to produce an attenuated strain. The freeze-dried virus was found to retain its immunizing value for at least 36 months if kept at 4°C and for 22 months if kept in the dark at room temperature. These are the maximum periods at which viability tests have been carried out and are not necessarily end-points.

When used as a vaccine this product has the advantage of producing a local eye reaction (Fig. 1) which demonstrates that the animal has been immunised. It is also cheap to prepare and, if used by the instillation method, it is safe. A susceptible dog, when vaccinated, will show the eye reaction whereas an immune animal will not. An animal in the incubative stage of the disease will not give the eye reaction if vaccinated more than 24 hours after exposure, but will develop a serious but not necessarily a fatal attack. Protection is acquired in the comparatively short time of 3 days. A non-reactor, presumably an immune animal, can be revaccinated without any danger. The only disadvantage of the use of the living HCC virus is the possibility of infecting in-contact, susceptible dogs. The present investigation indicated that a susceptible dog, when exposed to a vaccinated animal during the reaction, contracted the disease, but it is very unlikely that the danger-period lasts for more than 15 days. Considering that HCC-virus infection is no longer confined to any particular area or any continent, but is widespread all over the world, this method of vaccination is not likely to spread the disease further. It might be wise, however, to keep vaccinated dogs at home for about two weeks. The method gives a strong immunity and is, therefore, to be recommended in areas where the disease is endemic.

When animals were vaccinated with egg-adapted distemper virus-vaccine and exposed to fully virulent distemper infection by inoculation or contact before the minimum period necessary for conferring immunity had elapsed, a high mortality resulted. Protection was effective 3 days after subcutaneous inoculation but was delayed until 7 days after the conjunctival instillation of the distemper virus-vaccine. Apart from this there was no difference in response between dogs treated by the two methods. In the later experiments the two virus vaccines were pooled and given by conjunctival instillation because of an accident that once occurred when they were given separately. On this occasion three dogs were inoculated subcutaneously with the distemper vaccine by means of a syringe which had been used to prepare the HCC vaccine and to instill it into the eyes. As the result of the administration of small quantities of the HCC virus subcutaneously two puppies died and the other was seriously ill. By pooling the two vaccines and freeze-drying them together the risk of introducing HCC virus under the skin is avoided. When challenged by intravenous inoculation of fully virulent DCX+ HCC viruses, vaccinated dogs were found to be protected for at least a year; in addition, no breakdown has been reported in a group of

40 susceptible dogs, belonging to members of the staff which were vaccinated and reacted, and have been exposed to street infection for the last two years. DCX egg-adapted freeze-dried vaccine retained its protecting properties for at least 35 months if kept at 4°C.

Nineteen dogs which had been previously vaccinated against both infections after different intervals, did not show any eye reaction to further conjunctival instillation of HCC virus and remained unaffected. This showed that there would be no danger in accidental repeated vaccination by this method.

CONCLUSIONS

The successful results of dual vaccination against dog distemper (DCX) and hepatitis (HCC) viruses are reported. The HCC vaccine consisted of the fully virulent virus of Rubarth's disease, introduced by conjunctival instillation. The DCX vaccine consisted of egg-adapted distemper virus, fully attenuated, administered subcutaneously or mixed with HCC virus and instilled in the conjunctival sac. The two viruses may be freeze-dried, either separately or mixed together.

When administered simultaneously, no interference with the efficiency of the two virus-vaccines was observed.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Mr. J. R. Hudson for his advice and interest, and to Dr. A. W. Stableforth for his encouragement and permission to publish this work. Thanks are also due to Mr. A. A. King for technical assistance and to Mr. A. Chandler for looking after the experimental dogs. The Onderstepoort Strain of DCX was kindly supplied by Dr. Haig.

REFERENCES

Flint, J. C. (1953). *J. Amer. vet. med. Ass.*, **122**, 110.

Gillespie, J. H. Robinson, J. I., and Baker, J. A. (1952). *Proc. Soc. exp. Biol. N.Y.*, **81**, 461.

Haig, D. A. (1948). *Onderstepoort, J.vet. Sci.*, **23**, 149.

Mansi, W. (1951). *Brit. vet. J.*, **107**, 214; (1953). *J. comp. Path.*, **63**, 236; (1955). *Ibid.*, **65**, 291.

[*Received for publication, September 29th, 1955*]

THE SYNERGISM BETWEEN PYRIMETHAMINE AND SULPHADIMETHYLPYRIMIDINE IN THE CONTROL OF *EIMERIA TENELLA*

By

S. B. Kendall and L. P. Joyner

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

In 1954 Lux reported the coccidiostatic activity of a group of 2, 4—diaminopyrimidines and of some structurally related dihydrotriazines. From his published experimental data it did not appear likely that any of these compounds would by themselves compare in efficiency with the sulphonamides already in common use for the control of coccidiosis in domestic animals. Lux further reported, however, a synergistic effect which was particularly apparent when 2, 4—diamino-5-chlorophenyl-6-ethyl-pyrimidine (pyrimethamine) was used in combination with some sulphonamides.

At Weybridge this observation was extended with a view to developing effective measures for the control of caecal coccidiosis caused by *Eimeria tenella* in chickens and the marked synergism observed between sulphadimethylpyrimidine (sulphadimidine) and pyrimethamine was briefly reported (Joyner and Kendall, 1955). It was suggested that the activity of the drugs in combination was such that their use was likely to lead to a marked advance in the chemotherapy of *E. tenella*.

The present paper further considers the experimental data on which this conclusion was based.

MATERIALS AND METHODS

During the course of observations designed to evaluate the use of pyrimethamine and sulphadimidine, young R.I.R. or W.L. chicks, previously isolated as far as possible from the risk of adventitious infection, were inoculated orally with sporulated oöcysts of *E. tenella* in numbers sufficient to cause a heavy rate of mortality among untreated birds. The birds were maintained throughout the experimental period in electrically heated wire-floored brooders. On each occasion the success of treatment was judged by comparing the rate of mortality with that which occurred among groups of untreated chicks kept under identical conditions.

In each instance treatment commenced at the 48th hour following infection and was continued for three days, by which time all birds in the untreated groups were in the final stages of the disease and many had died. Drugs were given mixed in the food, medicated meal being substituted for the ordinary diet (a proprietary chick mash) during the experimental period.

RESULTS

Table 1 and Table 2 record the daily mortality (calculated as a percentage of the total number of birds in each group) of chicks infected with *E. tenella* and treated from the second to the fifth day following the day of infection (Day 0 in the Tables) with: (a) sulphadimidine, at concentrations in the food of 0.05 per cent or 0.1 per cent; (b) pyrimethamine, at concentrations of 0.005 per cent or 0.01 per cent; or (c) the two compounds in combination.

TABLE 1

DELAYED MORTALITY IN CHICKS INFECTED WITH *E. TENELLA* AND TREATED WITH SULPHADIMIDINE AND PYRIMETHAMINE IN COMBINATION

Day	*Treatment*				
	0·05% sulphadimidine	*0·01% pyrimeth-amine*	*0·1% sulphadimidine and 0·01% pyrimethamine*	*0·05% sulphadimidine and 0·01% pyrimethamine*	*None*
0	Day of infection				
0+5	65	24			81
0+6	26	17			19
0+7		4			
0+9			30	27	
0+10			40	43	
0+11			7		
Total % mortality	91	45	77	70	100

It will be seen that none of the treatments recorded in Table (1) was of great value although on this occasion pyrimethamine (at a concentration of 0.01 per cent) had some therapeutic effect. The two drugs in combination appeared to have little effect on the rate of mortality. By contrast, however, full therapeutic control was effected when the two drugs were used in combination in a second test, the concentration of sulphadimidine in the food having been reduced to 0.05 per cent and that of pyrimethamine to 0.005 per cent (Table 2).

Judged solely on the final rate of mortality it appeared, therefore, that reducing the concentration of pyrimethamine increased the activity of the sulphonamide. It will be noted, however, that the use of the higher concentration of pyrimethamine (Table 1) was followed by mortality which occurred at a different time from that observed with the untreated disease. As shown in both Table 1 and

Table 2 mortality as the result of untreated disease occurred principally on the fifth and sixth days following infection (Day 0+5 and Day 0+6).

TABLE 2

PERMANENT CONTROL OF *E. TENELLA* AS THE RESULT OF TREATMENT WITH SULPHADIMIDINE AND PYRIMETHAMINE IN COMBINATION

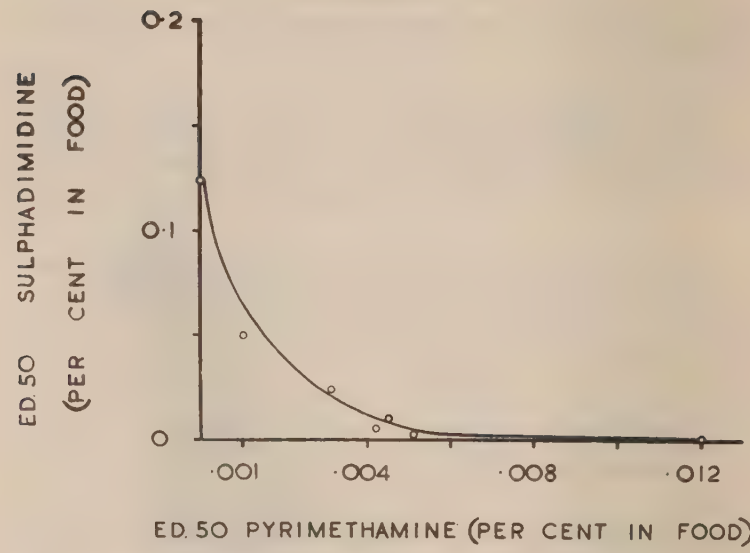
Day	*Treatment*				
	0·1% sulphadimidine	*0·01% pyrimeth-amine*	*0·005% pyrimeth-amine*	*0·05% sulphadimidine and 0·005% pyrimethamine*	*None*
0	Day of infection				
0+5	50	21	42		23
0+6	21	29	21		46
0+7					8
Total % mortality	71	50	63	0	77

Treatment with 0.01 per cent pyrimethamine in conjunction with 0.1 per cent or 0.05 per cent sulphadimidine (Table 1) apparently caused a temporary inhibition in development of the parasite with subsequent mortality following withdrawal of the drug. This effect was similar to that described by Kendall and McCullough (1952) when high concentrations of sulphadimidine were used in the treatment of *E. tenella*. It was concluded that the drug interfered with the development of immunity upon which the ultimate survival of the birds depends. This resulted in a higher mortality among those birds receiving drug in excess of the normal therapeutic dose.

By analogy, therefore, it appeared that the combination of sulphadimidine and pyrimethamine possessed a potency against *E. tenella* comparable with exceedingly high doses of the sulphonamide alone. It seemed likely that therapeutic activity could be demonstrated with much lower doses. This conclusion warranted a more extensive investigation of the effects of sulphadimidine and pyrimethamine in combination at different concentrations.

In order to observe in greater detail the potentiation of sulphadimidine by pyrimethamine a series of assays was carried out to determine the dose of pyrimethamine necessary to protect 50 per cent of infected chicks receiving, simultaneously, known subeffective doses of sulphadimidine. Three-point assays were made using groups of at least 12 chicks, each of the three groups receiving different concentations of pyrimethamine in food containing a fixed proportion of sulphadimidine. The 50 per cent protection

FIG. 1.



Showing the 50 per cent protection points of different concentrations of pyrimethamine and sulphadimidine.

points (E.D.50) were obtained graphically using the probit transformation to obtain linearity. The points obtained from a series of such experiments were plotted as shown in Fig. 1.

Rollo (1955) has used this method to demonstrate the potentiation of sulphadiazine by pyrimethamine in the control of *Plasmodium gallinaceum* in chicks and he has shown that potentiation is indicated by the points on the curve falling below the straight line joining the E.D.50 of the two drugs given alone. Figure 1 therefore gives clear evidence of very strong potentiation between sulphadimidine and pyrimethamine in their effects on *Eimeria tenella*, because the E.D.50 of pyrimethamine alone was found to be 0.012 per cent in the food while that of sulphadimidine was 0.12 per cent. When used in combination however, the concentrations of both drugs could be reduced to approximately 1/5 to obtain an equivalent effect.

DISCUSSION

The observations recorded in this paper followed the published work of Lux (1954) who investigated the possibility that potent inhibitors of *Eimeria tenella* might be found among compounds, other than sulphonamides, which interfered with the para-aminobenzoic acid—folic acid metabolic sequence.

Until recently, in all fields of medicine, the search for new therapeutic agents has been essentially empirical and involved

the screening of enormous numbers of compounds of which all but a few were likely to prove ineffective. This situation has changed in certain directions, however, with the establishment of theoretical bases for the use of chemical substances specifically designed to cause an interference with selected metabolic reactions of pathogenic micro-organisms (Medical Research Council, 1953-1954). Among the most noteworthy advances has been the elucidation of the part which the sulphonamides play in interfering with the production of the essential metabolite closely similar to themselves, namely p-aminobenzoic acid. Also of major importance has been the preparation of synthetic compounds, notably the diaminopyrimidines, which are designed to act as antagonists to folic acid.

Because the sulphonamides and the diaminopyrimidines are believed to act separately and sequentially in the metabolic pathway which leads to the synthesis, within the pathogen, of nucleic acid, it could be inferred that members of the two groups in combination were likely to act in synergistic fashion. This effect was, in fact, illustrated and elucidated by Goodwin and Rollo (1955) and by Rollo (1955) during the course of studies on *Plasmodium gallinaceum*. Our observations on the synergistic effect of sulphadimidine and pyrimethamine have shown that the potentiation curve (Fig. 1) is closely analgous to that illustrated by Rollo (1955) for pyrimethamine and sulphadiazine acting on *P. gallinaceum*. This suggests that the metabolism of *E. tenella* is very similar to that of the plasmodia, a belief which is strengthened by the evidence of Lux (1954) who showed that a group of dihydrotriazines (closely related to the potent antimalarial metabolite of proguanil) were also effective against *E. tenella*, both alone and in synergistic combination with sulphaquinoxaline.

The implication is, therefore, that both p-aminobenzoic acid and folic acid are essential to the metabolism of *E. tenella*, in particular to the development of the second generation schizont, the growth of which involves the synthesis of nuclear material in exceptionally large amounts.

CONCLUSIONS

Young chickens, experimentally infected with *Eimeria tenella*, were given a mixture of 0.01 per cent pyrimethamine and 0.1 per cent or 0.05 per cent sulphadimidine in the food. A marked but temporary inhibition in the development of the parasite was observed, followed by acute disease after the cessation of treatment.

By contrast, treatment with 0.005 per cent pyrimethamine together with 0.05 per cent sulphadimidine produced full therapeutic control.

A high degree of synergism was indicated by a series of assays to determine the 50 per cent protection points with different proportions of the two drugs.

REFERENCES

Goodwin, L. G., and Rollo, I. M. (1955). *Biochemistry and Physiology of Protozoa* Vol. II Ed. Hutner, S.H. and Lwoff A.; New York.
Joyner, L. P., and Kendall, S. B., (1955). *Nature, London,* **176,** 975.
Kendall, S. B., and McCullough, F. S. (1952). *J. comp. Path.,* **62,** 116.
Lux, R. E. (1954). *Antibiotics and Chemotherapy* IV, 971.
—— Medical Research Council, 1953-1954. *Annual Report* H.M. Sta. Off; London.
Rollo, I. M. (1955). *Brit. J. Pharmacol.,* **10,** 208.

[*Received for publication, September 30th,* 1955]

THE CHEMOTHERAPY OF EXPERIMENTAL *ERYSIPELOTHRIX RHUSIOPATHIAE* INFECTION IN CHICKS AND TURKEYS

By

H. Williams Smith

*Animal Health Trust, Houghton Grange, Huntingdon**

INTRODUCTION

Penicillin is the agent most commonly employed in the treatment of *Erysipelothrix rhusiopathiae* infection in turkeys. One disadvantage of penicillin from the economic point of view is that more than one dose is usually needed to produce a cure (Van Es, Olney and Blore, 1945; Gifford and Jungherr, 1946; Grey, 1947a; Brown, Doll, Bruner and Kinkaid, 1949; Prier and Alberts, 1950). Aureomycin is less effective than penicillin (Prier and Alberts, 1950). Streptomycin has been shown experimentally to have some protective value when administered before infection (Grey, 1947b). The sulphonamides are valueless in the treatment of this disease. Recently, Hamilton (1954) has reported on the treatment of experimental *E. rhusiopathiae* infection in adult turkeys with NN[1]-dibenzylethylenediamine dipenicillin (benzathine penicillin), a form of penicillin possessing a prolonged action. Using turkeys weighing 27.4 to 29.5 lb., he found that 200,000 units given up to 72 hours before infection protected them against death. When 100,000 units were injected as a single dose, 17 to 26 hours after infection, 2 of 9 turkeys survived compared with 4 of 9 when the dose was increased to 200,000 units. The experimental disease was uniformly fatal in untreated turkeys.

The purpose of this paper is to report on the value of penicillin, procaine penicillin, benzathine penicillin, streptomycin, chloramphenicol, aureomycin, terramycin and furazolidone in the experimental form of *E. rhusiopathiae* infection. These agents were first tested *in vitro* and then on infected one-day-old chicks. Those agents that appeared most promising were finally studied in infected turkeys.

MATERIALS AND METHODS

Chicks. Unsexed one-day-old White Leghorn chicks (approx. weight 40 g.) were employed.

Turkeys. These were of the Broad Breasted Bronze breed and were used in experiments at 10 to 12 weeks of age when they weighed approximately 1½ to 2 kg.

E. rhusiopathiae *cultures.* Six strains of *E. rhusiopathiae*, 1224, 1694, 3406, 6332, 7999 and 8163, obtained from the National Collection of Type Cultures, Colindale, London were used in the in vitro sensitivity tests. All infection experiments were carried out with strain 7999.

*Present address: Lilystone Hall, Stock, Essex.

In vitro sensitivity tests. A suitable range of two-fold serial dilutions of each chemotherapeutic agent was made in 5 ml. of nutrient broth. Each series of tubes and a control tube containing medium but no agent was then inoculated with approximately 100,000 bacteria of a broth culture of *E. rhusiopathiae*. The tubes were incubated at 37°C for 24 hours and read. The lowest concentration of the agent that completely inhibited bacterial multiplication, as judged by visual examination, was recorded as the minimal inhibitory concentration. In the tests with sodium sulphamezathine the sulphonamide-inhibitors were first removed from the nutrient broth by the method of Evans (1948) and the size of the bacterial inoculum was reduced to approximately 100 bacteria per tube.

Chemotherapeutic agents. The aqueous penicillin employed was crystalline sodium penicillin G of a potency of approximately 1,600 units per mg. The procaine penicillin was suspended in a mixture of sodium carboxymethylcellulose and a sorbitan mono-oleate polyoxalkylene derivative ('Distaquaine'). Benzathine penicillin, maintained as a sterile dispersable powder, was suspended in distilled water for injection. All forms of penicillin were expressed in terms of international units. In comparative tests with other agents, the amounts of the penicillin preparations used were selected to correspond with those of the other agents, working on the basis of 1 mg. of sodium penicillin G containing 1,600 units. Streptomycin was employed as the sulphate and aureomycin and terramycin as the hydrochloride. Terramycin hydrochloride was used suspended in mineral oil as well as in aqueous solution. Chloramphenicol was used in powder form for oral administration and in a soluble form (Allen and Hanbury) for injection. All agents given by mouth were dispensed in gelatin capsules. Furazolidone was also given mixed in the food.

Method of producing infection. Chicks were infected by the intramuscular injection of 0.1 ml. of a 24 hour broth culture of strain 7999, containing approximately 100 million viable *E. rhusiopathiae* bacteria per ml. The intravenous route was employed in turkeys, the dose being 0.2 ml.

The examination of organs of chicks and turkeys for E. rhusiopathiae. Approximately 4 g. of the liver, the whole of the spleen and a loopful of bone marrow from each bird were dispersed in 25 ml. of nutrient broth, pH 6.8, containing 1:1,000 sodium azide and 1:100,000 crystal violet (Packer, 1943) and incubated at 37°C for 24 hours. This material was then streaked on to plates of bovine blood agar which were incubated at 37°C for 24 hours and examined for the presence of *E. rhusiopathiae*.

RESULTS

The in vitro Sensitivity of E. rhusiopathiae *to Chemotherapeutic Agents*

The sensitivity of six strains of *E. rhusiopathiae* to seven different chemotherapeutic agents is illustrated in Table 1. Penicillin was the most active tested, 0.05 to 0.1 units/ml. being sufficient to inhibit the growth of all six strains. These strains were also very sensitive to terramycin (0.25 to 0.5 μg./ml), aureomycin (0.5 to 1.0 μg./ml.) and furazolidone (0.5 to 2.0 μg./ml.). They were moderately sensitive to streptomycin (2.0 to 4.0 μg./ml.) and chloramphenicol (2.0 to 4.0 μg./ml.), and completely resistant to sodium sulphamezathine.

TABLE 1

THE IN-VITRO SENSITIVITY OF SIX STRAINS OF *E. RHUSIOPATHIAE* TO CHEMOTHERAPEUTIC AGENTS

Agent	*Minimal inhibitory concentration (μg./ml.)* for strain number*					
	1224	*1694*	*3406*	*6332*	*7999*	*8163*
Penicillin	0·1	0·05	0·05	0·1	0·1	0·1
Terramycin	0·25	0·25	0·25	0·5	0·5	0·5
Aureomycin	0·5	1·0	0·5	1·0	1·0	1·0
Furazolidone	0·5	0·5	1·0	1·0	1·0	2·0
Streptomycin	2·0	4·0	2·0	2·0	2·0	4·0
Chloramphenicol ..	2·0	4·0	2·0	4·0	4·0	4·0
Sodium Sulphamezathine	>1000	>1000	>1000	>1000	>1000	>1000

*The results for penicillin are expressed in units/ml.

The Treatment of Infected Chicks

The administration of chemotherapeutic agents at intervals after infection. The results of administering chemotherapeutic agents, 25 mg./kg., twice daily, for three days, to chicks, commencing one day after infection, are shown in Table 2. The dose of procaine penicillin employed was 40,000 units/kg., this being equivalent in potency to 25 mg./kg. of the penicillin preparation used. Terramycin, procaine penicillin and penicillin, all given intramuscularly, were the most satisfactory agents studied. None of the chicks treated

TABLE 2

THE EFFECT OF ADMINISTERING CHEMOTHERAPEUTIC AGENTS
25 mg./kg.,* twice daily for three days, to chicks commencing one day after infection

Agent	Route	*No. of 25 chicks that died on the following days after infection*							*Total died*	
		2	*3*	*4*	*5*	*6*	*7*	*8-14*	*No.*	*%*
Terramycin ..	intra-muscularly	0	0	0	0	0	0	0	0	0
Procaine penicillin	,,	0	0	0	0	0	0	0	0	0
Penicillin ..	,,	0	0	0	2	1	0	0	3	12
Streptomycin ..	,,	0	0	4	11	1	1	0	17	68
Chloramphenicol	,,	11	7	3	3	0	0	0	24	96
Furazolidone ..	orally	3	14	5	2	0	0	0	24	96
Chloramphenicol ..	,,	19	6	0	0	0	0	0	25	100
None ..		20	5	0	0	0	0	0	25	100

*Procaine penicillin was administered at the rate of 40,000 units/kg., this being equivalent in potency to 25 mg./kg. of the penicillin that was used.

with streptomycin died during the treatment period but they appeared unwell and deaths occurred rapidly when treatment was discontinued. Furazolidone given orally and chloramphenicol given either orally or intramuscularly had very little effect upon the disease. The course of the disease in different batches of untreated

chicks was reasonably uniform, most chicks dying on the second day after infection and the remainder on the third.

The effect of administering the agents once, instead of twice, daily for three days is illustrated in Table 3. Furazolidone, streptomycin and chloramphenicol were not studied in view of the

TABLE 3

THE EFFECT OF ADMINISTERING CHEMOTHERAPEUTIC AGENTS

25 mg./kg.,* once daily, for three days to chicks commencing one day after infection

Agent	*Route*	*No. of 25 chicks that died on the following days after infection*							*Total died*	
		2	*3*	*4*	*5*	*6*	*7*	*8-14*	*No.*	*%*
Terramycin in oil ..	intramuscularly	0	0	0	0	0	0	0	0	0
Terramycin ..	,,	0	0	0	0	0	0	1	1	4
Benzathine penicillin	,,	0	0	0	0	1	1	1	3	12
Procaine penicillin	,,	0	0	0	0	1	0	3	4	16
Penicillin ..	,,	0	0	0	2	1	2	0	5	20
Terramycin ..	oral	13	4	3	1	0	0	0	21	84
Aureomycin ..	,,	15	7	1	1	0	0	0	24	96
None ..		22	3	0	0	0	0	0	25	100

*The penicillin preparations were administered at the rate of 40,000 units/kg., this dose being equivalent in potency to 25 mg./kg. of the penicillin that was used.

poor results obtained with them in the previous experiment. Instead, terramycin in oil and benzathine penicillin given intramuscularly and terramycin and aureomycin given orally were included. Best results were obtained with terramycin in oil, terramycin, benzathine penicillin, procaine penicillin and penicillin, all given intramuscularly. Very poor results were obtained with terramycin and aureomycin given orally.

When the chemotherapeutic agents, 25 mg./kg. or equivalent, were administered to chicks intramuscularly, one and three days after infection, a much greater mortality rate was observed in all cases than after terramycin, terramycin in oil and benzathine penicillin treatment being approximately half that after procaine penicillin and penicillin treatment (Table 4). The survival times of the chicks treated with terramycin, terramycin in oil and benzathine penicillin were very much longer than those treated with either procaine penicillin or penicillin, indicating that these three agents were persisting for much longer periods of time in the tissues, relapses occurring when they were no longer present in significant amounts. Somewhat similar results were noted when treatment was applied one and two, instead of one and three, days after infection.

When a single dose of chemotherapeutic agent, 25 mg./kg. or its equivalent, was given intramuscularly to infected chicks one day after infection (Table 5), the results were in keeping with those of the previous experiment; although the mortality rate was high

TABLE 4

THE EFFECT OF ADMINISTERING CHEMOTHERAPEUTIC AGENTS
25 mg./kg.,* to chicks intramuscularly, one and three days after infection

Agent	No. of 25 chicks that died on the following days after infection							Total died	
	2	3	4	5	6	7	8-14	No.	%
Terramycin	0	1	2	0	0	3	5	11	44
Terramycin in oil	0	0	2	0	2	2	6	12	48
Benzathine penicillin	0	0	0	2	2	2	6	12	48
Penicillin	1	13	6	0	0	0	0	20	80
Procaine penicillin	0	11	8	2	1	0	0	22	88
None	20	5	0	0	0	0	0	25	100

*The penicillin preparations were administered at the rate of 40.000 units./kg., this dose being equivalent to 25 mg./kg. of the penicillin that was used.

in all groups, much longer survival times were noted in those chicks that had been treated with either benzathine penicillin, terramycin or terramycin in oil than in those that had been treated with procaine penicillin or penicillin. Much better results were obtained when the single dose of chemotherapeutic agent was increased from 25 to 100 mg./kg. or its equivalent (Table 6). These results demonstrated the superiority of terramycin in oil and benzathine penicillin over terramycin, penicillin and procaine penicillin. Cultural examination of the organs of the survivors of the groups of chicks treated either with terramycin in oil or benzathine penicillin did not reveal any of them to be latently infected with *E. rhusiopathiae*.

TABLE 5

THE EFFECT OF ADMINISTERING A SINGLE DOSE OF CHEMOTHERAPEUTIC AGENT
25 mg./kg.,* intramuscularly to chicks one day after infection

Agent	No. of 25 chicks that died on the following days after infection							Total died	
	2	3	4	5	6	7	8-14	No.	%
Benzathine penicillin	0	1	7	2	1	2	4	17	68
Terramycin	0	0	4	7	3	2	2	18	72
Terramycin in oil	0	1	1	6	7	1	4	20	80
Penicillin	0	5	15	2	0	0	0	22	88
Procaine penicillin	0	8	13	3	0	0	0	24	96
None	21	3	1	0	0	0	0	25	100

*The penicillin preparations were administered at the rate of 40,000 units/kg., this dose being equivalent to 25 mg./kg. of the penicillin that was used.

The administration of a single dose of chemotherapeutic agent to chicks before infection. The effect of administering a single dose of either terramycin in oil, 100 mg./kg., or benzathine penicillin, 160,000 units/kg., at different intervals of time before infection is illustrated in Table 7. The protective action of benzathine penicillin was more prolonged than was that of terramycin in oil.

TABLE 6

THE EFFECT OF ADMINISTERING A SINGLE LARGE DOSE OF CHEMOTHERAPEUTIC AGENT

100 mg./kg.,* intramuscularly, to chicks one day after infection

Agent	*No. of 25 chicks that died on the following days after infection*							*Total died*	
	2	*3*	*4*	*5*	*6*	*7*	*8-14*	*No.*	*%*
Terramycin in oil	0	0	0	0	0	0	2	2	8
Benzathine penicillin	0	0	0	1	0	1	1	3	12
Terramycin	0	0	0	2	4	3	5	14	56
Penicillin	1	6	9	2	1	0	2	21	84
Procaine penicillin	0	0	17	4	0	0	1	22	88
None	13	12	0	0	0	0	0	25	100

*The penicillin preparations were administered at the rate of 160,000 units/kg., this dose being equivalent to 100 mg./kg. of the penicillin that was used.

The effect of the continuous feeding of mash containing furazolidone to chicks, commencing before infection. When a group of chicks fed mash containing 0.04 per cent. furazolidone from hatching time onwards were infected when three days old, no effect was noted on the course of the disease apart from a slight increase in survival time. The mortality rate was 96 per cent. compared with 100 per cent. in a similar group of chicks fed mash that did not contain furazolidone.

TABLE 7

THE EFFECT OF ADMINISTERING A SINGLE DOSE OF CHEMOTHERAPEUTIC AGENT TO CHICKS BEFORE INFECTION

Interval between preventive treatment and infection (days)	*No. of 10 chicks that died after preventive treatment with*		
	Benzathine penicillin (160,000 units/kg.)	*Terramycin in oil (100 mg./kg.)*	*None*
0	0	1	10
1	0	4	10
2	1	5	10
3	4	9	10
4	8	–	10

The Treatment of Infected Turkeys

For reasons of economy, only the value of the two agents that were most effective in the treatment of infected chicks, terramycin in oil and benzathine penicillin, were studied in turkeys. The results of treating groups of 16 turkeys with a single dose of either terramycin in oil, 100 mg/kg., benzathine penicillin, 160,000 units/kg., or benzathine penicillin, 16,000 units/kg., are shown in Table 8. The lower dose rate of benzathine penicillin was included in this investigation as it corresponded with the highest dose rate employed by Hamilton (1954). All three forms of treatment were

TABLE 8

THE TREATMENT OF TURKEYS

One intramuscular injection of chemotherapeutic agent, 20 hours after injection

Treatment	*No. of 16 turkeys that died*
Terramycin in oil, 100 mg./kg.	0
Benzathine penicillin, 160,000 units/kg. ..	0
Benzathine penicillin, 16,000 units/kg. ..	0
None	15

completely effective in controlling the disease. Four survivors from each group were subjected to re-infection 20 days after treatment. None of them showed any signs of clinical infection. The organs of the remaining birds in these three groups were examined for the presence of *E. rhusiopathiae* with completely negative results.

DISCUSSION

The results in chicks indicate that, although several agents given in multiple dosage were effective in the control of *E. rhusiopathiae* infection, only terramycin in oil and benzathine penicillin were effective when given as a single dose. Although the value of these two agents was confirmed in turkeys, it is noteworthy that benzathine penicillin was effective in a much lower dose rate in turkeys than in chicks. This is probably a reflection of the greater susceptibility of chicks to fatal infection and to the slow rate at which they develop immunity compared with turkeys. The results with benzathine penicillin were better than those obtained by Hamilton (1954). This may be merely due to the fact that the type of infection he produced was more severe.

Since erysipelas is a very much more serious economic problem in swine than in turkeys, the present work indicates that terramycin in oil and benzathine penicillin may be worthy of extensive trial in the treatment of the disease in swine.

It is interesting that, although *E. rhusiopathiae* was sensitive to furazolidone *in vitro*, this agent did not control the experimental disease in chicks. Furazolidone has also been shown to be valueless in the treatment of poultry suffering from either experimental *Pasteurella septica* infection (Smith, 1955) or experimental *Mycobacterium tuberculosis* infection (Smith, unpublished work). It may well be that, as far as bacterial infections in poultry are concerned, the use of this agent will be limited to the treatment of salmonella infections in which it is of proven value.

CONCLUSIONS

Erysipelothrix rhusiopathiae was very sensitive *in vitro* to penicillin, terramycin, aureomycin, and furazolidone, moderately sensitive to streptomycin and chloramphenicol, and completely resistant to sodium sulphamezathine.

One single dose of either terramycin in oil, 100 mg./kg., or benzathine penicillin, 160,000 units/kg., intramuscularly, was effective in the treatment of experimental *E. rhusiopathiae* infection in chicks. Terramycin, procaine penicillin and penicillin intramuscularly, were also effective in multiple dosage. Streptomycin and chloramphenicol given intramuscularly and chloramphenicol, terramycin, aureomycin and furazolidone given orally were of little value.

One single dose of either terramycin in oil, 100 mg./kg., or benzathine penicillin, 16,000 units/kg., cured turkeys experimentally infected with *E. rhusiopathiae*. Treated turkeys were completely resistant to re-infection and their organs were not found to be latently infected with *E. rhusiopathiae*.

ACKNOWLEDGMENTS

I am grateful to Mr. W. E. Crabb, A.I.M.L.T. for his capable technical help. I also wish to express my sincere thanks to Dr. R. F. Gordon for providing the facilities for carrying out this work and to Dr. S. T. Cowan of the National Collection of Type Cultures, Colindale, for providing the cultures of *E. rhusiopathiae*. The aureomycin was kindly supplied by Lederle Laboratories, the benzathine penicillin by I.C.I. Ltd., the chloramphenicol by Allen and Hanburys Ltd., the furazolidone by A. J. White, Ltd., the streptomycin by Glaxo Ltd. and the terramycin by Pfizer Ltd.

REFERENCES

Brown, R. G., Doll, E. R., Bruner, D. W.. and Kincaid, A. S. (1949). *J. Amer. vet. med. Ass.*, **114**, 438.
Evans, R. J. (1948). *J. Hyg., Camb.*, **46**, 422.
Gifford, R., and Jungherr, E. (1946). *Mich. State. Coll. Vet.*, **7**, 18.
Grey, C. G. (1947a). *Vet. Med.*, **42**, 74; (1947b). *Ibid.*, **42**, 216.
Hamilton, C. M. (1954). *Poult. Sci.*, **33**, 629.
Packer, R. A. (1943). *J. Bact.*, **46**, 343.
Prier, J. E., and Alberts, J. O. (1950). *Ibid.*, **60**, 139.
Smith, H. Williams (1955). *J. comp. Path.*, **65**, 309.
Van Es, L., Olney, J. E., and Blore, I. C. (1945). *Rev. Bull. Neb. agric. Exp. Sta.*, No. 141, 15.

[*Received for publication, October 7th*, 1955]

BACTEROIDES INFECTION IN KANGAROOS

By

P. S. Watts and Sibely J. McLean

Institute of Medical and Veterinary Science, Adelaide, and the Department of Bacteriology, University of Adelaide, South Australia

INTRODUCTION

In 1953 Tucker and Millar recorded a mycotic disease of marsupials in Brisbane characterised by swelling of the face. They reported the isolation of *Nocardia* spp. from the lesions and in the course of their report they fully discussed the literature which will therefore not be further considered here.

The condition they described corresponded closely with one we have often encountered in kangaroos (*Macropus rufus* and *M. giganteus melanops*), wallabies (*Macropus agilis*) and Euros (*Macropus robustus*) at the Adelaide Zoological Gardens. The condition, which has long been known, has been regarded as a fatal infection. From occasional bacteriological examinations we had concluded that the causal organism was a *Fusiformis* spp. but in view of Tucker and Millar's article further work was carried out.

RESULTS

Clinical Histories

The clinical condition was a single, localised, thick-walled abscess containing yellowish-green pus, usually more or less inspissated and adherent to the walls of the cavity. The commonest site was the soft tissues at the side of the upper jaw, level with the first molar teeth, and a slight swelling in this area was often the first sign of the disease noticed by the animal attendant. The prognosis in these cases was good if treatment was instituted. Other sites were also occasionally affected. Lesions on the front lips and gums responded to treatment, but if they occurred between the rami of the lower jaw or involved the maxillary or mandibular bones the prognosis was not good.

Treatment consisted of the injection of 1 mega unit of aqueous penicillin into and around the site of infection followed by daily intramuscular injection of 600,000 units of procaine penicillin. This treatment needed to be instituted early as otherwise the animal tended to cease feeding, after which it was difficult to get it to start eating again.

When the reaction at the site had subsided and the lesion had regressed to a relatively static condition, usually after 7 to 10 days, the animals were anaesthetised by the intravenous injection of Thiopentone Sodium. It was customary to inject about 5 ml. of a 5 per cent. solution, i.e. 0.25 g., of drug into an anterior vein of the hind leg and then to continue slowly till reflexes ceased. It was rarely necessary to exceed 0.5 g., however large the kangaroo. The

animals recovered well from this treatment and several females carrying young in the pouch did not evict them. When anaesthesia was complete the infected area was widely opened by two incisions crossing at right angles. The four flaps so formed were reflected and the free corners removed to give a wide drainage area. The cavity was curetted and all pus and necrotic tissue removed. It was then packed with sulphapyridine and the wound left open. It was dressed for a few days thereafter to prevent too rapid healing. Despite the extensive wound thus produced in many cases, few animals ceased feeding and in nearly all these the bone was involved.

Bacteriological Investigations

From the deeper layers of pus cultures were made on horse blood agar plates and in cooked meat media. Two blood plates and one cooked meat medium were inoculated with the material from each lesion; one blood plate was incubated aerobically for 24 hours and the other was incubated in an anaerobic jar for 2 to 3 days. After 2 days incubation the cooked meat medium was plated on blood agar. In every kangaroo an anaerobic Gram-negative rod was detected, frequently accompanied by other bacteria. The types of bacteria found are set out in Table I.

TABLE 1

BACTERIA ISOLATED FROM LESIONS IN KANGAROOS

Animal	*Site of infection*	*Bacteria isolated*	
		Anaerobes	*Aerobes*
K1	Side of maxilla	Gram-negative rod.	Nil.
608	Side of maxilla	Gram-negative rod. Streptococcus.	Staphylococcus. Diphtheroid.
K2	Pouch (same animal as 608)	Gram-negative rod. Gram-negative cocco-bacillus. Gram-positive coccus.	Gram-negative rod.
K3	Side of maxilla	Gram-negative rod. Gram-positive coccus.	Nil.
K4	Side of maxilla	Gram-negative rod. Gram-negative cocco-bacillus	Nil.
K5	Side of maxilla	Gram-negative rod. Gram-negative cocco-baccillus. Gram-positive coccus.	Nil.

The anaerobic Gram-negative rods from K1 and K2 had rounded ends. The rods from K1 were approximately 1.6μ x 0.3μ and those from K2 were approximately 2.1μ x 0.3μ. The colonies which were approximately 0.2 mm. diameter at 48 hours, were greyish-white, translucent and entire, and had irregular, speckled or concentric markings. They were similar to those described by Lewis

and Rettger (1940) for *Bacteroides vulgatus* and to the photographs of the colonies of *Fusiformis nucleatus* and *Fusiformis polymorphus* given by Hine and Berry (1937). The Gram-negative rods from 608 were longer, more slender and had pointed ends ; their colonies also had irregular markings. The colonies of the Gram-negative rods from K3 and K4 were like those of K1 and K2 except that they had no markings. The colonies of the rod from K5 were very transparent and also had no markings. Although morphologically all these bacteria except those from 608 could be described as coccobacillary we prefer to regard them as true rod-shaped i.e. as bacillary types.

The anaerobic Gram-negative cocco-bacilli isolated from K2, K4 and K5 appeared to be identical. They could be distinguished from the small rod-shaped organisms by their very small size. They were difficult to measure accurately but seemed to be about half the size of the rods described above. On blood agar they produced small colonies which etched into the surface of the medium producing "pock-marks". These colonies were very characteristic and were identical with those described by Beveridge (1941) for *Fusiformis nodosus*. However the morphology of the organisms was entirely different from that of *F. nodosus*; they were minute cocco-bacilli while *F. nodosus* is a large rod characterised by the presence of terminal enlargements.

Both the bacilli and the cocco-bacilli were similar morphologically and culturally to the organisms described by various authors as *Fusobacterium* (*Fusiformis*) or *Bacteroides* (Slanetz and Rettger, 1933; Hine and Berry, 1937; Spaulding and Rettger, 1937; Lewis and Rettger, 1940; Beveridge, 1941). In Bergey's Manual of Determinative Bacteriology, 1948, the genus *Bacteroides* was described as cells with rounded ends and the genus *Fusobacterium* as cells with pointed ends. The Gram-negative anaerobes from the kangaroos belonged to the genus *Bacteroides* except for that from 608 which appeared more closely related to *Fusobacterium*. A detailed study of these organisms is now in progress.

An anaerobic Gram-positive coccus which was also isolated from kangaroo 608, occurred in chains and resembled a streoptococcus. Anaerobic Gram-positive cocci, recovered from K2, K3 and K5, occurred in groups and clusters suggestive of staphylococci.

DISCUSSION

In human infections members of the *Bacteroides* genus have been isolated either alone or associated with other organisms. Thus Beigelman and Rantz (1949) found that *Bacteroides* were frequently isolated in pure culture but the concurrent recovery of anaerobic streptococci was of significant frequency. McVay and Sprunt (1952) reported 7 infections where *Bacteroides* were isolated in association with an anaerobic streptococcus and one case where Bac-

teroides were associated with *Staph. aureus, E. coli, A. aerogenes* and unidentified diphtheroids. Fisher and McKusick (1953) found anaerobic streptococci in 5 out of 14 cases of *Bacteroides* infections. It would appear that the organisms found in the kangaroo lesions were similar to those isolated from human *Bacteroides* infections and differed from the aerobic Nocardia group found by Tucker and Miller (1953) in their marsupial series.

CONCLUSIONS

Lesions at the side of the maxilla in kangaroos are described which were treated successfully with penicillin and sulphapyridine. Although several bacterial species were isolated from some of these lesions, in each case an anaerobic Gram-negative rod was present. In one case the organism was closely related to the genus *Fusobacterium* while in the others the bacilli were provisionally identified as *Bacteroides*.

REFERENCES

Beigelman, P. M., and Rantz., L. A. (1949). *Arch. int. Med.*, **84**, 605.
Beveridge, W. I. B. (1941). *Aust. Council sci. indust. Res. Bull.*, 140.
Fisher, A. M., and McKusick, V. A. (1953). *Amer. J. med. Sci.*, **225**, 253.
Hine, M. K., and Berry, G. P. (1937). *J. Bact.*, **34**, 517.
Lewis, K. H., and Rettger, L. F. (1940). *Ibid.*, **40**, 287.
McVay, L. V., and Sprunt, D. H. (1952). *Ann. int. Med.*, **36**, 56.
Slanetz, L. W., and Rettger, L. F. (1933). *J. Bact.*, **26**, 599.
Spaulding, E. H., and Rettger, L. F. (1937). *Ibid.*, **34**, 535.
Tucker, R , and Miller, P. (1953). *J. comp. Path.*, **63**, 143.

[*Received for publication, November 28th, 1955*]

ERRATUM

R. F. W. Goodwin and Ruth Saison, Vol. 66 No. 2,

Page 165 For "Fig. 1" read "Fig. 2".

THE BLOOD GROUPS OF THE PIG

III. A Breed Difference in Iso-Antibody Response After Vaccination With Crystal Violet Swine Fever Vaccine

By

R. F. W. Goodwin and Ruth Saison*

Department of Veterinary Clinical Studies, Madingley Road, Cambridge

INTRODUCTION

Crystal violet swine fever vaccine can stimulate the production of red cell iso-antibodies when injected into pigs (Goodwin, Saison and Coombs, 1955). Primary evidence for this was obtained by injecting pigs experimentally but it was also shown that among vaccinated pigs in the field there was a high percentage of animals with iso-antibody titres of 128 or more, whereas unvaccinated pigs rarely possessed serum iso-antibodies and then only at very low titres. As no unvaccinated sow has, in our experience, destroyed her offspring with iso-antibodies, it has been concluded that there is a direct association between vaccination and haemolytic disease. During the early investigations into this disease, however, certain differences were observed between breeds of pigs and these differences have since been examined more closely.

The first breed factor concerned those sows in the field that had had litters clinically affected with haemolytic disease. On reviewing our first twenty cases it was striking that the sow was always either of the Essex or Wessex breed. These breeds are popular and they provide a major part of the breeding sows in this country. But the Large White breed of pig is similarly popular and it seemed significant that no cases of haemolytic disease had been reported to us during the same period from the many Large White herds in this area. The second factor was related to the boars that sired affected litters for in every case they were of the Large White breed. Such a uniformity among the sires might have had a simple explanation in that, given that the sow must be an Essex or Wessex pig, the Essex or Wessex x Large White mating is extremely common. Alternatively, the piglet produced by this mating, being largely white, demonstrates more obviously than the pure Essex or Wessex piglet the main symptoms of haemolytic disease. Nevertheless, the observation that the sires were always of one breed and the dams always of another suggested an important breed difference and warranted further investigation.

At the same time an indication was obtained that, whereas there might be marked differences between breeds, there might be considerable uniformity within one particular breed. During investigations in the field it was not unusual for an owner who had lost piglets with haemolytic disease, and who possessed one or more

*From the Ontario Veterinary College, Canada.

Large White boars other than the one that sired the affected litter, to ask whether an alternative boar would likewise produce affected litters. On titrating the serum of the sow against these alternative cells, it was commonly observed that, theoretically, the other boars in the herd would also be unsuitable. It was questionable, therefore, whether the antigenic structure of red cells from Large White boars varied in such a way that only a few boars could sire litters affected with haemolytic disease. Examples of these titrations using the red cells from alternative boars are given later.

The work described here set out mainly to compare the incidence of iso-antibodies among Large White and Essex/Wessex sows, when the cells used for testing were from the appropriate breeding boars. While the latter red cells were available, however, it was convenient to explore concurrently the question of the similarity in red cell antigenic structure among Large White boars.

MATERIALS AND METHODS

Pigs. The animals from which blood samples were obtained, together with the manner of their selection, are described in detail with the results. In brief, however, the serum iso-antibody titres of 97 Essex/Wessex sows have been compared with the corresponding titres for 69 Large White sows. Secondly, following upon the observations on the titres shown by alternative Large White boars in a few herds, titrations have been made using two powerfully haemolytic sera and the cells of 20 Large White boars.

Blood samples and serological tests. The collection of serum and red cell samples, together with the techniques used to determine the presence of iso-antibodies or to place the animals into the A or O blood groups have been described elsewhere (Saison, Goodwin and Coombs, 1955; Goodwin *et al.*, 1955). During this work, however, the earlier good fortune, whereby boars of only group O were encountered, did not hold.

Among the Large White herds, it was possible to use cells from a group O boar in all but one herd where the three breeding boars were of group A (herd N). In this case, four out of the ten sows sampled were of group O and could not, therefore, be tested against any local boar. They were tested instead against the next available group O boar.

In the Essex/Wessex herds the boars were of group O in all but two cases. In herd J the boar supplying the testing cells was of group A but all the sows were similarly of group A. The other exception was in herd H, but although the three sows were of group O and the testing cells of group A, the sera registered generally similar titres with cells from a group O boar.

RESULTS

*Serum Iso-antibodies in Two Breeds of Vaccinated Pigs**

Essex/Wessex pigs

Two thirds of the serum samples from Essex and Wessex pigs were obtained during the investigation of cases of haemolytic disease

*The difference between the Essex and Wessex breeds appears to be so trivial that they are best considered as one breed in this context and will be discussed as such. Thus the two breeds compared are the combined Essex/Wessex breed and the Large White breed.

in the field. On visiting a herd in which haemolytic disease had arisen it was the practice first to bleed the sows that had had clinically affected litters and then to bleed a number of other sows to see how the iso-antibody titres varied within a cross-section of the herd. The results using the sera from those sows whose litters were said to be unaffected have been recorded here. The iso-antibody titrations for the sows with clinically affected litters have not been incorporated, because it is felt that they were specially selected for high titres. The remaining Essex and Wessex serum samples were from four vaccinated herds (C, D, E and G) in which haemolytic disease had not been observed. Altogether, in the ten Essex/Wessex herds from which samples were obtained there were 97 sows that had been vaccinated twice or more but had not been reported to us as having piglets affected with haemolytic disease.

In all but one herd the sera were titrated against the cells of the local boar. The exception was a herd that had had four sows with litters affected with haemolytic disease (herd K). Four Large White boars were in use but no attempt was made by the owner to note the boar responsible for each litter. It was impracticable to titrate all the samples from this herd against all four boars and hence the cells of pig 57 were used instead. These cells were from a group O pig whose selection has been described elsewhere (Goodwin *et al.*, 1955) and they seemed capable of detecting readily the serum iso-antibodies in Essex/Wessex sows. Some results for pig 57 and the local boars in this herd are compared in Table 5. In all herds where the local boar's cells were used, the boar was of the Large White breed except in herd D where an Essex boar was used.

FIG. 1

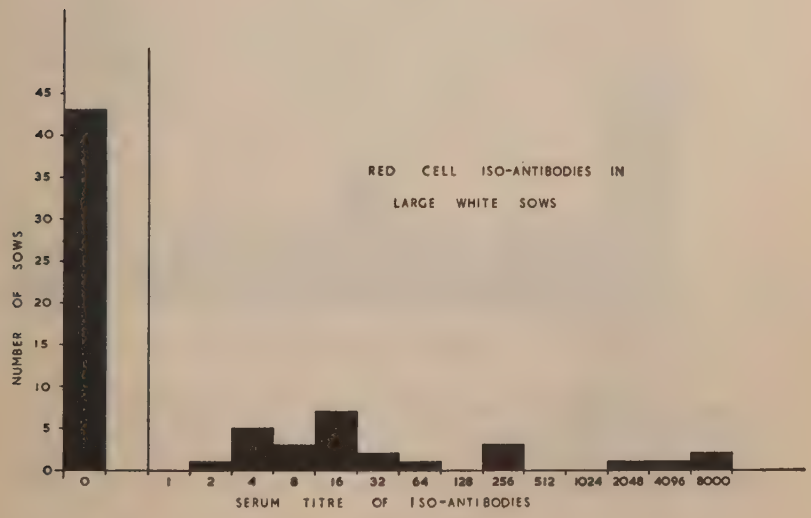


TABLE 1

RED CELL ISO-ANTIBODIES IN ESSEX AND WESSEX SOWS

Herd	*Number of sows*	*Distribution of sows according to iso-antibody titres*											*Cells used for titrations**
		0	1	2	4	8	16	32	64	128	256	512 *or greater*	
A	25	—	2	1	1	5	6	2	2	3	1	2	Large White boar
B	3	—	—	—	—	—	1	—	—	1	—	1	Large White boar
C	17	—	—	2	1	—	1	2	1	2	1	7	Large White boar
D	7	—	—	2	—	1	1	—	—	1	—	2	Essex boar (1)
E	5	1	—	—	1	—	2	—	—	—	1	—	Large White boar (21)
F	1	—	—	—	—	—	—	—	—	—	—	1	Large White boar
G	2	—	1	—	1	—	—	—	—	—	—	—	Large White boar
H	3	—	—	—	—	—	1	1	1	—	—	—	Large White boar
J	13	4	2	1	1	2	—	1	—	1	—	1	Large White boar (3)
K	21	—	—	1	1	—	2	2	2	2	4	7	Pig 57
Totals	97	5	5	7	6	8	14	8	6	10	7	21	

*In both Table 1 and Table 2 the figures in brackets refer to the number of the boar if it appears in Table 6.

Page 167 For "Fig. 2" read "Fig. 1".

The distribution of iso-antibody titres among Essex/Wessex sows is shown in Figure 1. The number of samples obtained, together with the distribution of iso-antibody titres and other information for each herd separately is given in Table 1. The titres shown were obtained with the indirect sensitization test.

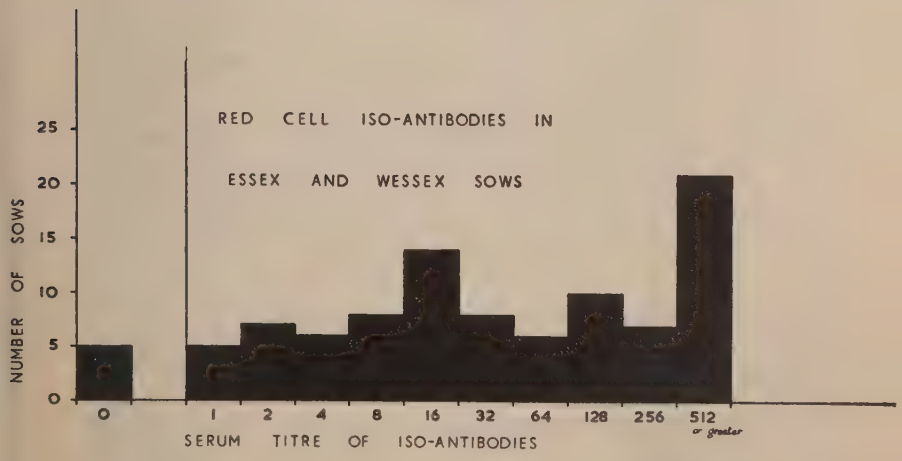
Large White pigs

The sera of 69 Large White sows were next examined to see whether they also contained iso-antibodies as a result of vaccination. Large White herds were sought where the sows had been vaccinated several times and while all had been vaccinated twice, there were 42 sows that had been vaccinated thrice or more, including 24 that had been injected four or more times.

In all cases the cells of a Large White boar were tested against these sera and, as has been described earlier, it was possible to use a local boar for all but four titrations. As it had been decided to bleed no more than eight to twelve sows in each herd and thereby obtain a wider breed sample, eight herds were visited to obtain these sera and cells from eight different Large White boars were used in the titrations.

The distribution of iso-antibody titres among the Large White sows is shown in Figure 2, while the more detailed information applicable to each herd is given in Table 2. Again, the titres recorded were obtained with the indirect sensitization test.

FIG. 2



In only one herd (herd O) were the titres of iso-antibody found to be very high and even in this herd four out of the twelve serum samples showed no detectable iso-antibody. This herd was unusual, however, in that the sows were bled fourteen days after vaccination.

TABLE 2

RED CELL ISO-ANTIBODIES IN LARGE WHITE SOWS

Herd	*Number of sows*	*Distribution of sows according to iso-antibody titres*											*Cells used for titrations*
		0	2	4	8	16	32	64	256	2048	4096	8000	
C	1	—	1	—	—	—	—	—	—	—	—	—	Large White boar
M	8	5	—	—	—	2	1	—	—	—	—	—	Large White boar (9)
N	10	8	—	2	—	—	—	—	—	—	—	—	Large White boar (12)†
O	12	4	—	1	—	—	1	—	2	1	1	2	Large White boar (13)
P	10	9	—	1	—	—	—	—	—	—	—	—	Large White boar (15)
Q	10	7	—	—	2	1	—	—	—	—	—	—	Large White boar (18)
R	8	3	—	1	—	2	—	1	1	—	—	—	Large White boar (20)
S	10	7	—	—	1	2	—	—	—	—	—	—	Large White boar (19)
Totals	69	43	1	5	3	7	2	1	3	1	1	2	

† Four sera from this herd were titrated against Large White boar (18)

Further titrations using the same red cell and serum samples taken nine weeks later showed generally lower antibody levels (Table 3) and it appears that the original high values resulted from the proximity of vaccination.

TABLE 3

DECLINE IN ANTIBODY TITRES IN HERD O

Sow (*Large White*)	*Time after vaccination*	
	14 Days	*11 weeks*
182	256	—
279	256	256
281	2048	512
282	4096	—
290	8000	256
315	8000	128

Titrations using the Cells of Different Large White Boars

Alternative sires for sows in the field

Some examples are given first of titrations that were made using the cells of original boars and the most readily available alternative boars against the sera of sows that had had piglets affected with haemolytic disease in the field (Table 4). The sows having clinically affected litters in each herd have been marked with a single asterisk. Both Tables 4 and 5 record the titres registered by the indirect sensitization test only.

Table 5 presents separately the titrations that were made with the sera from the four sows in herd K. It has been mentioned that four boars were in use in this herd but mating records were not kept. Although the sera from the remainder of the herd were titrated only against the cells of pig 57, the sera from the four clinical cases were titrated against all the boars' cells as well as against pig 57. The object here was to try and identify the boar or boars responsible for the haemolytic litters. The high titre registered by boar 1 and sow 266 could be due to the presence of anti-A in the serum of this particular sow.

With few exceptions, the titrations of each serum against more than one sample of cells gave titres that were within a tube of each other. In many instances the titres were identical. This suggested that the boars that could sire affected litters were not uncommon and the question of uniformity among Large White boars was then approached in another way.

Recognition of antibodies in two haemolytic sera by a panel of boars' cells

In setting out to compare the incidence of iso-antibodies in Large White and Essex/Wessex herds, the main concern was that some factor might be overlooked that favoured the high percentage of

animals with iso-antibodies among the Essex and Wessex pigs. One such factor could have been associated with the fact that many of the Essex/Wessex samples had been obtained from herds in which cases of haemolytic disease had arisen. It was possible that these affected litters were observed not only because there were sows with the necessary serum iso-antibodies but because the boars in those herds

TABLE 4

UNSUITABILITY OF ALTERNATIVE BOARS AS SHOWN BY ISO-ANTIBODY TITRES

Herd (*Essex/Wessex*)	*Sow*	*Titre using cells of*	
		Original sire (*Large White*)	*Alternative sire* (*Large White*)
A	Sue*	128	128
	Primrose*	256	256
	Bluegate 148*	128	128
B	Large White**	—	—
	1*	128	128
	104	4	8
	W	>512	>512
H	15	64	32
	17	16	8
	41*	1024	1024†
	42*	512	512†
	205	32	<2
J	22	8	16
	30	4	4
	36*	512	512
	202	2048	1024
	222	1	2
	277	—	2
	318	128	2048

*These sows had litters clinically affected with haemolytic disease.

**The only Large White sow in the table.

†Yet another sire was tested against these sera without hope of a satisfactory mating: the titres for sows 41 and 42 being once more 1024 and 512 respectively.

TABLE 5

ANTIBODY TITRES USING THE CELLS OF FOUR LARGE WHITE BOARS AND PIG 57 AGAINST THE SERA FROM FOUR ESSEX SOWS WITH LITTERS AFFECTED WITH HAEMOLYTIC DISEASE

Sow	*Group*	*Boar 1* (*Group A*)	*Boar 2* (*Group O*)	*Boar 3* (*Group O*)	*Boar 4* (*Group O*)	*Pig 57* (*Group O*)
266	O	8000	512	256	1024	1024
119	O	512	256	256	1024	1024
271	O	256	128	64	256	256
187	O	512	256	128	256	1024

possessed particularly suitable red cells. In circumstances such as these, to reject only the titrations using sera from the dams of affected litters would give a false sense of security, for the remaining sera in the herd would also be titrated against these specially selected cells.

As a check on this possibility, the red cells of the Large White boars in the Pedigree Large White herds were tested against two sera obtained from an Essex and a Wessex sow. These sera were chosen because they were from sows that afflicted their litters with a rapidly fatal form of haemolytic disease. The serum of the Wessex sow (R1) had an antibody titre of 2048 against the cells of the Large White boar that sired her last affected litter. This sow was of group A. The Essex sow (D42) was of group O and there was a possibility, therefore, that a group A boar would detect the anti-A antibody if it were present. This serum was judged to be particularly suitable, however, because the iso-antibody titre against the cells of the boar (group O) that sired her last affected litter was only 512.

Table 6 shows the results of the titrations (direct agglutination and indirect sensitization) made with the two reference sera and

TABLE 6

TITRATION OF TWO REFERENCE SERA AGAINST THE CELLS OF VARIOUS LARGE WHITE BOARS AND ONE ESSEX BOAR

Boar	*Group*	*Serum R1 (Wessex)*		*Serum D42 (Essex)*	
		D.A.	*I.S.T.*	*D.A.*	*I.S.T.*
1 (Essex)	O	128	8000	16	1024
2 (L.W.)	O	32	>2048	8	512
3 ,,	A	128	>8000	8	*512*
4 ,,	A	128	1024	4	*256*
5 ,,	O	<16	256	16	256
6 ,,	O	<16	1024	8	256
7 ,,	O	64	2048	8	512
8 ,,	A	128	4096	8	*256*
9 ,,	O	128	4096	8	256
10 ,,	A	<32	1024	4	*128*
11 ,,	A	64	1024	4	*128*
12 ,,	A	64	1024	8	*256*
13 ,,	O	64	256	<4	64
14 ,,	A	64	1024	4	*128*
15 ,,	O	<32	1024	<4	128
16 ,,	A	<32	2048	4	*128*
17 ,,	O	<32	1024	8	128
18 ,,	O	64	2048	4	128
19 ,,	O	<32	1024	4	256
20 ,,	O	32	1024	4	256
21 ,,	O	<16	4096	4	512

D.A.—Direct agglutination test
I.S.T.—Indirect sensitization test
Titres obtained with serum D42 and the cells of group A boars (I.S.T.) are in italics.

includes results obtained with the cells of boars from other than Large White herds. Boar 1 was the only boar that was not a Large White and this animal was the Essex boar that had been encountered when

collecting sera from the Essex and Wessex pigs. Boars 2 to 7 were various Large White boars in Essex/Wessex herds from which cells were obtained during this period. Boars 8 to 20 were the boars bled in Large White herds. They include all the group O boars whose cells were used to provide the results in Figure 2, plus other group O and group A boars in the same herds whose cells were not used except against these two sera. The total number of twenty Large White boars came from ten different herds.*

Considering serum R1, it can be seen that sixteen out of the twenty Large White boars gave titres within one tube of the base-line titre of 2048. Two gave higher titres and only two gave obviously lower titres (both at 256). For serum D42, where 512 was the titre in its own herd, twelve boars gave titres within one tube of 512 while seven others gave a titre of 128. The remaining boar registered a titre of 64 and was one of the boars whose cells gave a low titre with serum R1. The titres obtained when serum D42 was used with those boars' cells that were of group A are shown in italics in Table 6, and it would appear that anti-A, if present, did not complicate the titrations with this serum. It is interesting that boar 13, which gave the lowest titres for both reference sera, was the boar that registered very high titres in one of the Large White herds (herd O).

The above evidence suggested that most of these Large White boars could sire litters that would succumb to haemolytic disease from the sows R1 and D42. To prove this point in practice would involve an extensive breeding programme but sow D42 has since been mated with one of the boars in Table 6 that had not previously sired an affected litter (boar 9) and all but three pigs from this mating died from acute haemolytic disease. Similarly another sow (D41) that had lost her entire litter with haemolytic disease was mated with a Large White boar that was unselected on its breeding history, for it had likewise not previously sired an affected litter. The titration of the boar's cells against serum D41 suggested that the litter would die as before and this, in due course, proved to be correct. Furthermore, D41 has recently farrowed again and the litter, which was this time sired by boar 9, was almost completely destroyed by haemolytic disease. With two separate sows, therefore, the results of the laboratory titrations were substantiated in practice and there is reason to believe that the many boars in Table 6 would also behave in accordance with their respective titrations if they were mated with sows R1 and D42.

The cells of boar 21 (Large White) in Table 6—also known as the Madingley boar—were titrated against the reference sera for a special reason. During our previous work these cells appeared poorly reactive (Goodwin, *et al.*, 1955) and this fact contrasted markedly

*In one herd a Swedish Landrace boar was available as a young replacement boar and it is worth recording that his cells titrated against the sera R1 and D42 gave titres of 1024 and 64 respectively in the indirect anti-globulin sensitization test.

with the uniformity displayed by the other Large Whites in Table 6. However, on testing the cells against the two reference sera it was clear that they were not unusual. The antibodies that we had stimulated experimentally, therefore, were in the main different from those present in two severe cases of haemolytic disease and this was probably due to the fact that all the pigs (such as 25 and 29) used for experimental injections in our earlier work were sired by the Madingley boar.

Analysis of Results

Previously, we had examined serum samples to detect the presence of iso-antibodies *per se*, and for this purpose any testing cells that could recognize a wide range of antibodies were suitable (Goodwin, *et al.*, 1955). In this work, however, we sought to identify incompatible matings and we were thus limited to testing the sera of breeding sows against the cells of local breeding boars. The sera of 145 breeding sows have been titrated in this way. A further 21 samples (herd K) were titrated against a standard testing cell instead of the local boars' cells but Table 5 shows that this cell did not detect iso-antibody at a generally higher titre. Hence the marked difference in the incidence and concentration of iso-antibodies between the Essex/Wessex herds and the Large White herds (Figures 1 and 2) indicates why several litters were affected with haemolytic disease in the herds of the first group and none was reported to be affected in the second. This explanation does not depend upon whether the two groups are representative samples of their breeds. Before attempting broader conclusions, however, it is important to consider whether Figures 1 and 2 truthfully depict a difference that exists between all heavily vaccinated pigs of the two breed groups.

Several arguments can be advanced to suggest that the Essex/Wessex sample is not representative. The first is that the red cells of the boars in Pedigree Large White herds were less able to recognize antibodies. This point has been discussed earlier and Table 6 showed that all but one of these boars could recognize iso-antibodies readily in two powerfully haemolytic sera. The boar that was exceptional (boar 13) did detect iso-antibody in the same two sera but at a titre two and three tubes lower respectively. It so happened, however, that boar 13 detected iso-antibody at a high titre in several sows in its own herd. Since it has been shown that the cells of alternative Large White boars could detect, with equal facility, iso-antibodies in Essex/Wessex herds, regardless of whether the sera originated from sows that had lost litters with haemolytic disease or not (Table 4), and that the boars in the Large White herds readily detected the iso-antibodies in haemolytic sera (Table 6), it is most probable that these latter boars would likewise have detected the iso-antibodies in the sera of Essex/Wessex sows that had not destroyed their piglets. In other words, had the cells of the boars in the Large White herds been used for the Essex/Wessex titrations they

would have given results very similar to those obtained with the cells of the local boars. This observation on the antigenic similarity of Large White boars has an obvious practical value in those herds where haemolytic disease has arisen.

It could next be argued that even though the results for all those sows whose litters developed frank haemolytic disease were excluded, the remaining sows were still selected because many animals in one herd might be similarly highly responsive to vaccination or, alternatively, the batch of vaccine that initiated cases of haemolytic disease might be particularly antigenic. Some guidance on this question may be obtained by dividing all the Essex/Wessex herds into two samples according to whether they were or were not brought to our notice because they were experiencing litters with haemolytic disease. Out of 63 samples from herds with haemolytic disease, 43 per cent had titres of 64 or more, 35 per cent had titres of 128 or more, 25 per cent had titres of 256 or more and 17 per cent had titres of, or greater than, 512. The corresponding figures for the 31 samples from herds without haemolytic disease are 48, 41, 35 and 29 per cent, so that at every stage the clinically unaffected herds had a higher incidence of iso-antibodies. Unless these latter herds were not comparable with the former herds in yet another way, it would seem that in herds where haemolytic disease occurs clinically there is no general tendency for the remainder of the herd to show an unusual incidence of iso-antibodies at high titre.

Disparity in the frequency of vaccination could be a further factor that might unbalance the sampling in the two groups and Table 7

TABLE 7

FREQUENCY OF VACCINATION IN BREED SAMPLES

Number of vaccinations	*Number of sows in each category*	
	Essex/Wessex herds	*Large White herds*
Two	37	27
Two or more	28	—
Three	16	11
Three or more	—	7
Four	7	4
Four or more	—	13
Five	—	2
Six or more	9	5
Totals	97	69

shows the distribution of animals according to the number of injections that they had received. A final comparison cannot be made without knowing more precisely the vaccination history of those Essex/Wessex sows that were said to have been vaccinated twice or more, but the percentage of Large White sows that had received three or more injections is considerably higher than the corresponding percentage of Essex/Wessex sows and it is almost certain, therefore, that vaccination was no more intensive in the latter group as a whole.

The next cause of distortion in the two breed samples could be due to dissimilarity in the times elapsing between vaccination and collecting the sera. The question of the maintenance of antibody titre will be discussed more fully elsewhere (Goodwin and Saison, in preparation) but in many instances vaccination produces only a short-lived antibody response: a sharp peak occurs about two weeks after injection and thereafter the titre declines fairly rapidly with time. It follows that if the Essex/Wessex samples were obtained nearer to vaccination, then the titres might be correspondingly higher. On analysing the results, it appears that the Essex/Wessex sample was in fact favoured in this respect (Table 8). However, by selecting from these results samples from the two breeds comparable

TABLE 8

COMPARISON OF INTERVALS BETWEEN VACCINATION AND SAMPLING FOR TWO BREED GROUPS

Time between vaccination and bleeding	*Percentage of total breed sample*	
	Essex/Wessex herds	*Large White herds*
Under 2 months	14	27·5
Under 3 months	60	43·5
Under 6 months	87	56·5

for the interval between vaccination and bleeding, it can be shown that the incidence of iso-antibodies remains considerably higher in the Essex/Wessex group. Furthermore, it is striking that among the sera of pigs bled within three months of vaccination, over fifty per cent of the Large White samples were devoid of antibodies whereas none of the Essex/Wessex samples was of this type. Thus, after considering this objection, there is still a great difference in iso-antibody response between Essex or Wessex sows and Large White sows, albeit that this difference is not characterized precisely by our results.

The other evidence for a true lack of iso-antibodies in vaccinated Large Whites is convincing but, unfortunately, only few animals are involved. In each of six different Essex/Wessex herds a serum sample was obtained from a Large White pig that had been vaccinated at

least twice. These sera titrated against the cells of the respective local boars gave one titre of 2, three others of less than 2 (which could have been negative) and two that were negative. Thus six Large White sera subject to the same conditions as the main Essex/Wessex herds gave results in keeping with the sample obtained from Large White herds.

Hence it is concluded that the difference between the two breed groups is real, and the absence of fatal cases of haemolytic disease among Large Whites can be partly understood. It is not clear whether the Large White breed is free from risk with the present vaccine, for although iso-antibodies were detected at high titre in some Large White sows these antibodies might differ from those generally producing haemolytic disease and also be less pathogenic in their effect. However, even if the iso-antibodies in Large White sera were powerfully haemolytic the apparent absence of cases of haemolytic disease among Large White sows could still be explained, for the number of Essex/Wessex sows brought to our notice as having piglets affected clinically with haemolytic disease is still less than thirty and the much lower incidence of iso-antibodies among Large Whites would result in clinical cases but rarely.

DISCUSSION

Some consideration can now be given to a theoretical explanation of the above findings. It is probable that the Large White breed possesses almost universally one or more antigens not generally existing among Essex/Wessex pigs. These antigens could also be particularly antigenic, although not necessarily so for it is possible that the Large White breed might develop equally high titres of antibody to Essex/Wessex antigens and this reverse situation remain masked because Essex/Wessex boars are not, in practice, mated to Large White sows. The antigens involved in haemolytic disease are probably not confined to the Large White breed for the disease has been seen in the offspring of an Essex sow when the litter was sired by an Essex boar. Furthermore, this Essex sire registered titres with two powerfully haemolytic sera above the average shown by Large White boars with the same sera (Table 6, boar 1), while the cells of a Large White boar known to detect iso-antibody at high titre in Essex pigs could barely improve upon the titres registered by this Essex boar against the sera of four sows possessing antibodies at high titre in its own Essex herd.

The vaccine most commonly used in Britain is supplied by the Veterinary Laboratory, Weybridge, and this vaccine was probably used on all the herds sampled here except herd R, where it is now known that a foreign vaccine was used instead. The Weybridge vaccine has been produced either from the so-called 'Blue' pigs (the offspring of the Essex/Wessex x Large White Cross) at one centre, or from Large Whites and their near crosses at another,

except for 26 and 11 per cent of the total issue in 1953 and 1954, respectively, which was imported from abroad. (No attempt has been made to identify the breeds of pigs associated with these foreign vaccines.) Taking the vaccine as a whole, therefore, it would contain mainly antigens possessed by the Essex/Wessex breeds and the Large White breed, with perhaps a predominance of the latter. During the manufacture of one batch of vaccine, care is taken to see that the treated whole blood is very well mixed and as the blood from about fifty pigs (less commonly splenic pulp is used) goes into each batch it is likely that the antigenic components are usually similar even though the relative quantities of the different antigens may vary.

If when the blood groups of pigs are characterized, it is found that the disease originates essentially from a breed difference in red cell antigenic structure and if it is then still necessary to continue using the existing vaccine (either because the lapinized vaccine has not reached a safe stage in development or has proved to have harmful side-effects) the possibility should be examined of reducing the risk in Essex/Wessex herds by producing some batches from Essex pigs alone. Even now, without waiting for further work, a case might be made for ceasing to inject Essex/Wessex pigs knowingly with the blood of Large Whites.

A breed difference in antigenic structure is not the only possible explanation of these results and, although it is less likely, it must be borne in mind that a completely different factor such as a poor ability on the part of Large Whites to maintain their antibody titres could greatly accentuate a much smaller difference in initial antibody response.

Our previous findings on the number of sows possessing iso-antibodies at high titre (Goodwin *et al.*, 1955) can now be reconsidered. Only three of the twice vaccinated sows bled in that work were not of the Essex or Wessex breed. Two of these exceptions were Large Whites and they registered very low titres but the third, a Large Black, provided a titre of 512. In the light of the present results, the original sample did not represent the vaccinated pig population as a whole and, as the Essex/Wessex and Large White herds provide a large part of the breeding sows in Britain, it is now clear that the over-all incidence of iso-antibodies that are recognized by the cells of breeding boars is much lower than originally indicated. This re-assessment of the number of breeding sows possessing iso-antibodies at high titres is the first step in the explanation that was sought for the great disparity between the incidence of sows theoretically at risk and the low mortality among piglets from haemolytic disease.

A re-examination of the control sera used in the original work is also necessary. The most important control sera were from 66 breeding sows. In this group, 28 were from Large Whites. It is fortunate, however, that all the remaining 38 sera were from Essex or Wessex sows. It may be significant that the few sera containing

antibody at low titre in this control group were all from Essex/Wessex sows. For the unvaccinated gilts and bacon pigs, out of 42 samples 7 were from Essex/Wessex animals, 14 from the Welsh breed and 21 from the so-called 'Blue' pigs. The animals mentioned in the Addendum to the same paper were also of the Blue type, except for 7 that were the offspring of a Middle White x Large White mating and 12 that were Pedigree Essex animals. It is known that the Blue type of pig can produce iso-antibodies after vaccination that would be detected by the cells used for the original titrations (pig 57) as all the pigs that we had vaccinated experimentally (Goodwin, *et al.*, 1955) were also of this type.

CONCLUSIONS

Among 97 Essex/Wessex sows, vaccinated twice or more with crystal violet vaccine, iso-antibodies were almost universal and often at a high titre. Among a seemingly comparable sample of 69 Large White sows, iso-antibodies were uncommon and indeed absent in two thirds of the samples. The red cells used for testing were those of local, mainly Large White, boars.

The red cells of alternative breeding boars (Large White), titrated against the sera of Essex/Wessex sows in the same herd, gave for each serum sample titres that were essentially the same, regardless of whether the serum was from a sow that had lost piglets with haemolytic disease or not. This similarity in antigenic structure was also observed when serum samples from two sows that afflicted their litters with acute haemolytic disease were each titrated against the cells of twenty Large White boars.

The theoretical and practical applications of these findings are discussed.

ACKNOWLEDGMENTS

One of us (R.S.) has enjoyed the hospitality and full facilities of Dr. R. R. A. Coombs's laboratory while working in Britain. Dr. J. C. Buxton and Mr. H. I. Field have notified us of cases of haemolytic disease in the field and thereby increased the material available for this study. Many of the samples from Large White sows were from the practices of Mr. F. G. C. Lawfield and Mr. W. A. Noble. Mr. P. J. D. V. Brett has rendered vigorous assistance in all matters concerned with handling and bleeding pigs. This work has been encouraged by Professor L. P. Pugh and has received the financial support of the Agricultural Research Council. Dr. A. W. Stableforth very kindly placed at our disposal complete details of the origins and production of the crystal violet vaccine that is supplied officially by the Veterinary Laboratory, Weybridge.

REFERENCES

Goodwin, R. F. W., Saison, R., and Coombs, R. R. A. (1955). *J. comp. Path.*, **65**, 79.

Saison, R., Goodwin, R. F. W., and Coombs, R. R. A. (1955). *Ibid.*, 71.

[*Received for publication, November 29th,* 1955.]

MYCOTIC DERMATITIS OF CATTLE IN BRITISH WEST AFRICA

By

K. S. Chodnik

Department of Animal Health, Gold Coast, West Africa

INTRODUCTION

The present report deals with a skin disease of cattle encountered in British West Africa which appears to resemble closely a disease reported from various parts of Africa under the name of cutaneous streptothricosis, (Curasson and Mornet, 1941; Curasson, 1942; Buck, 1948; Goyal, 1937; Beaton, 1933; Malfroy, 1938). On account of more urgent problems the prevalence and importance of this disease in West Africa may not have been fully appreciated: nevertheless, although the mortality in this disease is on the whole much lower than in major epizootic diseases, its incidence in some districts, and in certain seasons of the year, appears to be very high and constitutes an important problem in animal husbandry.

Whilst the disease is primarily one of cattle it has been encountered in goats (Hall, 1932; Hudson, 1937), sheep, and horses (Hudson 1937; Stableforth 1937). Van Saceghen (1915, 1916, 1932, 1934) has contributed a good deal of information on a form of contagious dermatitis of bovines in the Belgian Congo. Apart from the work of Hudson (1937) on a disease encountered in Northern Rhodesia, there is surprisingly little information about the condition in the reports dealing with the diseases in general encountered in West Africa (Best, 1927; Hall, 1932; Hornby, 1937). While the descriptions of clinical and gross pathological lesions are very similar the very varied bacteriological findings make it difficult to know whether the disease studied by various authors is the same entity.

Van Saceghen (1916) described a filamentous and coccoid organism which he named *Dermatophilus congolensis*, but later (1934) he concluded that the organism was a sarcina. In smears from the lesions, Best (1927) in Nigeria observed Gram-positive cocci, usually arranged in parallel rows and showing branching. Hudson (1937) in his studies on the organism isolated in Northern Rhodesia concluded that it is more likely to belong to the genus Actinomyces and proposed the name of *Actinomyces congolensis*.

PRESENT INVESTIGATION

This report deals with an investigation carried out on a disease encountered in the Gold Coast and Northern Nigeria. No attempt has been made to compare it with a similar disease encountered in other parts of Africa. During a visit to the Veterinary Laboratory in Vom, Northern Nigeria, a number of post-mortem examinations were performed on affected animals and material was collected for

both bacteriological and histopathological examination. It was possible later to continue this work in the Gold Coast and to confirm the identity of the disease in these two countries.

Clinical Picture

The disease under investigation is in most cases a chronic exudative dermatitis characterized by the formation of crusts. In the early stages moist round patches appear on various parts of the body which later become covered with a hard dry crust. Lesions range in size from very small nodule-like formations to large patches, and in the more advanced stages whole regions of skin may be covered with a thick crust. In the extreme cases which terminate in death almost the whole body is covered with lesions, which usually first appear on the sides and along the back. Isolated lesions occur round the mouth, on the ears, in the anal and perineal region and may resemble papillomata. There is a seasonal incidence which is related to the humidity, since in the dry season the condition either disappears entirely or passes into a chronic stage which flares up again in the next wet season with varying rapidity. Localized forms appear to have no serious effect. If the disease generalises there is a general debility, and lameness due to the formation of massive crusts along the legs; such cases are usually fatal. Apart from the slight irritation in the very early stages pruritis has not been noticed. Certain breeds of cattle, particularly the White Fulani, appear to be much more prone to the infection, but other breeds, whether pigmented or not, are by no means immune.

General Pathology

A variety of cutaneous lesions are encountered, ranging from mild forms which appear to cause little ill effect to acute, generalized forms which are fatal. By observing a large number of field cases and artificially infected animals it has been possible to follow the sequence of development of the lesions.

The earliest changes appear as patches of slight erythema which are visible only in unpigmented animals such as the White Fulani. In the next stage a small amount of exudate appears on the skin, giving the affected parts a greasy, matted appearance. The exudate coagulates to form a yellowish-brown crust which gradually changes into brownish flakes or hard scabs which adhere to the skin. The lesions are frequently of a creeping nature forming mosaic patterns or coalesce to produce round patches.

In regions of moderate humidity or where the rainy season is fairly short, the lesions seldom progress beyond this early stage and as a rule recede soon after the onset of dry weather, usually leaving patches of shorter hair and sometimes a greyish pigmentation of the skin. This form is frequently encountered in the Northern Territories of the Gold Coast where the rainy season is short and changes rather

abruptly into a dry season with semi-desert conditions. The incidence of the disease during the rains is very high but as these early stages do not appear to affect the health of the animals the disease remains almost unnoticed by the owners. Further progress of the disease depends, firstly, on prolonged high humidity and secondly on whether complications with other pathogenic organisms develop. In uncomplicated cases thick layers of a dry spongy material or hard button-like crusts are formed which project above the surface of the coat (Pl. I, Figs. 1 to 3). While the spongy type is usually diffuse and covers large areas, particularly of the regions where the coat is long (neck, legs), the hard button formations occur more frequently on the regions where the coat is shorter especially along the back, rump, face and perineal regions. By careful manipulation the dry masses can be detached from the epidermis without much damage, exposing a small quantity of moist exudate which dries fairly quickly to form a new crust. No pus is seen in such lesions. If a necrotic dermatitis develops, it usually spreads rapidly and covers areas of skin with a thick, purulent, offensively smelling exudate. This grave complication of mixed infection invariably terminates in death.

Bacteriological Examination

Staining properties of the microorganisms. Smears from the lesions reveal coccoid elements arranged in filamentous chains which are Gram-positive. The Gram-Glyn or Gram-Weigert methods of staining give the best results. The staining of mycelia differs slightly in the lesions and in cultures. In the lesions mycelia are scarcely Gram-positive and stain better with fuchsin and eosin, whereas in cultures they stain weakly by Gram's method and have no affinity for fuchsin or eosin. The microorganism is not acid fast.

Isolation and cultural characters. Material from 50 animals, including 15 from Northern Nigeria was examined culturally and included both the localized and the generalized forms of the disease. In the later stages localized lesions were usually selected since these yielded fewer contaminants. Cultures were made from the moist layer adhering to the epithelium after careful removal of the crust, as well as from the deeper parts of hard crusts which, as histological sections revealed, always contained masses of tangled mycelia. A variety of media was used and cultures were incubated under both aerobic and anaerobic conditions, and at incubator and room temperature. A mixed growth was invariably obtained and included staphylococci and streptococci. Every attempt was made to identify all the organisms isolated from the lesions. A mycotic organism closely resembling that invariably seen in the lesions on microscopical examination was recovered from all except four of the cases examined. It grew readily on ordinary peptone media, particularly when enriched with blood or serum and better on solid than in liquid media. Growth at laboratory temperature (25 to 35°C.) was much better than at 37°C. On primary cultivation at room tem-

perature the characteristic milk-white colonies did not usually appear before 3 to 5 days. On glucose agar a greyish-brown pigment was produced. In young colonies the surface layer was of a dry finely granular texture, while the deeper layer was more tenacious and adhered to the medium. In older cultures growth became leathery and tenacious so that whole colonies could be scraped off without disintegration. In liquid media a rather scanty and granular growth occurred on the surface and along the walls and easily sank to the bottom, leaving the supernatant clear. Microscopical examination of cultures showed a somewhat different appearance from that seen in sections of the lesions. The main surface mass of the colonies consisted exclusively of coccoid elements, while the thin layer adhering to the medium was richly interwoven with a filamentous mycelium. Removal of the colonies with the platinum loop left the mycelium adherent to the medium. On solid media the mycelia consisted of fine homogeneous filaments, whereas in liquid media coccoid elements appeared to be the only form of the organism produced.

On blood agar a clear zone of β haemolysis occurred around the colonies. In Loeffler serum the growth was not prolific. There was slow digestion of the medium marked by the formation of shallow crater-like depressions round the colonies after 7 to 12 days. On gelatin growth was rather scanty, liquefaction of the medium being complete in 4 to 6 days. On potato growth was scanty. There was no pigment formation.

Biochemical reactions. In litmus milk the medium was decolorized in 5 to 6 days, becoming pale pink. A fairly thick white pellicle was formed on the surface of the medium which remained liquid. The organism had very weak fermenting properties. A number of sugars showed a trace of acidity but always without gas formation. Fermentation of sugars was inconstant and irregular and some sugars including rhamnose, raffinose, laevulose, arabinose, and lactose, were unchanged. Nitrates were strongly reduced, there was production of H_2S and the catalase test was positive. Indol was not produced and the methyl red and Voges-Proskauer reactions were negative.

Histopathology

On histological examination the most striking feature is the superficial nature of the lesions which are restricted mainly to the upper layers of the skin and in particular to the epidermis. Even in advanced cases the deeper layers show little or no change.

The nature of the lesions is mainly exudative. In the early stages small amounts of exudate, mixed with a moderate number of polymorphonuclear leucocytes and some lymphocytes, appear at the base of the stratum corneum and form minute vesicles which cause separation of the cornified layer. Exudate escaping on the surface coagulates and dries to form crusts under which the process continues

as long as the lesion remains active. Continued moderate exudation, accompanied by desquamation, adds new layers to the hardening crusts. If this process continues for some time it results in the formation of either thick porous or hard cornified pyramidal masses (Figs. 1 to 3) which consist of dried exudate mixed with masses of desquamated epithelial cells, glandular secretions, and tangled masses of fungus.

The deeper layers, particularly the stratum granulosum, usually show a variable degree of infiltration with polymorphonuclear leucocytes and lymphocytes. Sometimes areas of proliferation and occasional vesiculation may be seen in the outer layers of the stratum granulosum, but it is not until the necrotic changes set in that destruction of the stratum granulosum results in exposure of the corium. Lesions which penetrate the corium are seen only when the hair follicles are invaded by the fungus, in which case a tangled mycelium appears to block the free passage of the glandular secretion, causing it to accumulate and distend the glandular ducts and lumen. Tissues in the vicinity of such hair follicles and adjoining glands are diffusely invaded by polymorphonuclear leucocytes.

Microscopical examination of preparations made directly from the lesions, including smears of exudate and histological sections of the surface crusts, are needed to obtain a complete morphological picture. Mycelial elements encountered in the lesions are characteristic. They consist of coccoid elements arranged in longitudinal symmetrical chains which may consist of a single row or of thick pillars of up to eight parallel rows of coccoid elements (Figs. 4 to 9). Occasionally asymmetrical and irregularly scattered cocci are seen in some parts of the filaments. Very fine mycelia do not show coccoid elements (Fig. 4). The cocci stain strongly Gram-positive, are very variable in size and are cemented together by a homogenous material which stains with eosin or fuchsin. Fully formed coccoid aggregates appear to be extremely fragile structures which break up easily into single cocci, or coccoid aggregates when films are made (Fig. 9). On closer examination of films from fresh exudate the whole cycle of development of the organism can be followed. Single coccoid elements which have reached a certain size (1 to 1.5 μ) give rise to a fine mycelium which at first is homogenous and Gram-negative. Later, fine Gram-positive coccoid granules arranged in symmetrical chains appear within the filaments and increase in size, expanding the matrix in which they have developed, until they form continuous rows which closely resemble chains of streptococci surrounded by a fine capsule.

Pathogenicity

Tests on small laboratory animals were mainly negative. Intraperitoneal and intravenous inoculation into rabbits and guinea-pigs did not produce any lesions. Fairly large subcutaneous doses caused the formation of a local abscess which ruptured and healed quickly.

Rubbing the organism into the skin of rabbits caused only a transient erythema lasting 2 to 4 days.

Experiments in cattle had to be carried out in the dry belt of the Gold Coast where the disease practically never progresses beyond the local lesions. In the coastal regions of the country where the disease is a real economic problem it is almost impossible to find native animals entirely free of lesions. Rubbing the organism into the healthy skin did not produce any lesions. When cultures were applied to the lightly scarified skin transient exudative lesions were induced, with scab formation resembling the early stages of the disease. If, however, the humidity was increased and maintained either by a local moist chamber or by frequent showers, localized lesions indistinguishable from the natural disease were reproduced and the organism could be recovered from such lesions. If continuous excessive humidity was maintained the lesions started to spread after 7 to 10 days. A total of ten animals was used in transmission experiments using pure cultures of the organism and in each case similar results were achieved. Two of the experimental animals in which the lesions receded a few weeks after the end of the experiment were returned to the herd and kept under observation. In both animals the lesions apparently passed into a dormant stage and recurred in the following wet season. They were used subsequently in the treatment experiment.

The results of our experiments show that typical lesions can be reproduced with pure cultures of the microorganism, and that humidity appears to be the only factor determining the course of the disease both under natural and experimental conditions.

Classification of the Microorganism

The organism isolated in culture is morphologically very similar to the one that is constantly found in smears and sections of the lesions. It has all the characteristics of the genus *Streptomyces,* belonging to the family *Streptomycetaceae* (Bergey, 1948). The colour of its mycelia, its proteolytic properties, the liquefaction of gelatin and the reduction of nitrates place it in the *Streptomyces albus* group. Waksman and Lachevalier (1953) favour group classification rather than the creation of new species. The present findings suggest that the term "Cutaneous Streptomycosis" would portray not only the clinical but also the microbiological nature of the disease.

DISCUSSION

The study of a skin disease of cattle in British West Africa shows that it is caused by a mycotic organism belonging to the *Streptomyces albus* group. The disease appears to be widespread and its incidence high, but it is only in some parts of West Africa where the climatic conditions allow the clinical lesions to develop that it constitutes a real economic problem. Climatic conditions are also responsible for its

seasonal appearance during the wet season and its total or partial disappearance during the dry season. There is evidence that the microorganism concerned leads a saprophytic life on the skin and that it is not until the equilibrium between the parasite and host is disturbed by extraneous factors that it becomes a true pathogen.

Two alternative possibilities of the mechanism of its pathogenicity can be postulated. Excessive multiplication of the microorganism under favourable conditions may result in invasion of the epidermis, or adverse climatic conditions, especially excessive humidity, may initiate a primary injury to the epidermis and thus create suitable conditions for rapid multiplication of the organism and the invasion of the epidermis. Whichever may be the explanation, it is the excessive humidity which stimulates the saprophyte to pathogenic action. Whether biting insects may also play a part in initiating the infection by causing skin injuries may also depend on the climatic conditions. No attempt was made to establish the relationship of the disease and the causal organism to the similar disease reported from other parts of Africa and the answer to this question would necessitate comparative bacteriological studies.

CONCLUSIONS

A description is given of the clinical symptoms, pathology and microbiology of a skin disease of cattle encountered in the Gold Coast and Northern Nigeria which closely resembles a condition reported from other parts of Africa under the name of cutaneous streptothricosis.

Cultural examination of 50 cases yielded a fungus which was identified as belonging to the *Streptomyces albus* group and which produced lesions like those of the natural disease when rubbed into the scarified skin of experimental cattle. In natural cases the causal agent was accompanied in the lesions by a variety of other organisms, including staphylococci and streptococci.

Further work is needed to determine the relationship of this disease to similar conditions described in other parts of Africa.

ACKNOWLEDGMENTS

My thanks are due to Dr. G. C. Ainsworth of the University of Exeter for the identification of the microorganism. I wish to thank also the Director of Veterinary Services for permission to publish.

REFERENCES

Beaton, W. G. (1933). *Annual Report Vet. Dept. Nigeria* 1932, Appendix B.
Bergey (1948). *Manual of Determinative Bacteriology* 6th edit. Williams and Wilkins Co; Baltimore.
Best, J. H. B. (1927). *Annual Report Vet. Dept. Nigeria* p. 52.
Buck, G. (1948). *Bull. Off. intern. Epiz.*, **29**, 117.

Curasson, G. (1942). *Traité de Pathologie Exotique vétérinaire et compareé* **2**, Vigot Freres; Paris VI.
Curasson, G., and Mornet, P. (1941). *Bull. Serv. Zoot. Epiz., A.O.F.*, **4**, 181.
Goyal, R. K. (1937). *Ann. Inst. Past. Paris*, **59**, 94.
Hall, G. N. A. (1932). *Annual Report Vet. Dept. Nigeria.*
Hornby, H. E. (1937). *Annual Report Dept. vet. Sci. and anim. Husb. Tanganyika Territory* 1934, p. 17.
Hudson, J. R. (1937). *Proc. R. Soc. Med.*, **30**, 55, 1457.
Malfroy, M. (1938). *Bull. Serv. Zoot. Epiz. A.O.F.*, **1**, 15.
Stableforth, A. W. (1937). *Proc. R. Soc. Med.*, **30**, 1455.
Van Saceghen, R. (1915). *Bull. Soc. Path. exot.*, **8**, 354; (1916). *Ibid.*, **9**, 97; (1932). *Agric. Elev. Congo belge*, **4**, 97; (1934). *Bull. Agric. Congo belge*, **25**, 590.
Waksman, S. A., and Lachevalier, H. A. (1953). *Guide to the Classification and Identification of the Actinomyces and their Antibiotics.* Williams and Wilkins Co; Baltimore.

[*Received for publication, September 12th, 1955*]

LEGENDS TO ILLUSTRATIONS

Fig. 1. Animal affected with cutaneous streptomycosis.

Figs. 2 and 3. Closer view of the lesions, showing horny scab formations.

Fig. 4. Preparation from agar culture, showing branching mycelium.

Figs. 5-8 Preparations from the skin scrapings, showing branching forms of streptomyces.

Fig. 9. From skin scrapings showing disintegration of coccoid chains. Note short fragments of remaining chain formations.

Photomicrographs showing streptomyces organism. Gram staining.

Figs. 4, 6 and 7– x 450

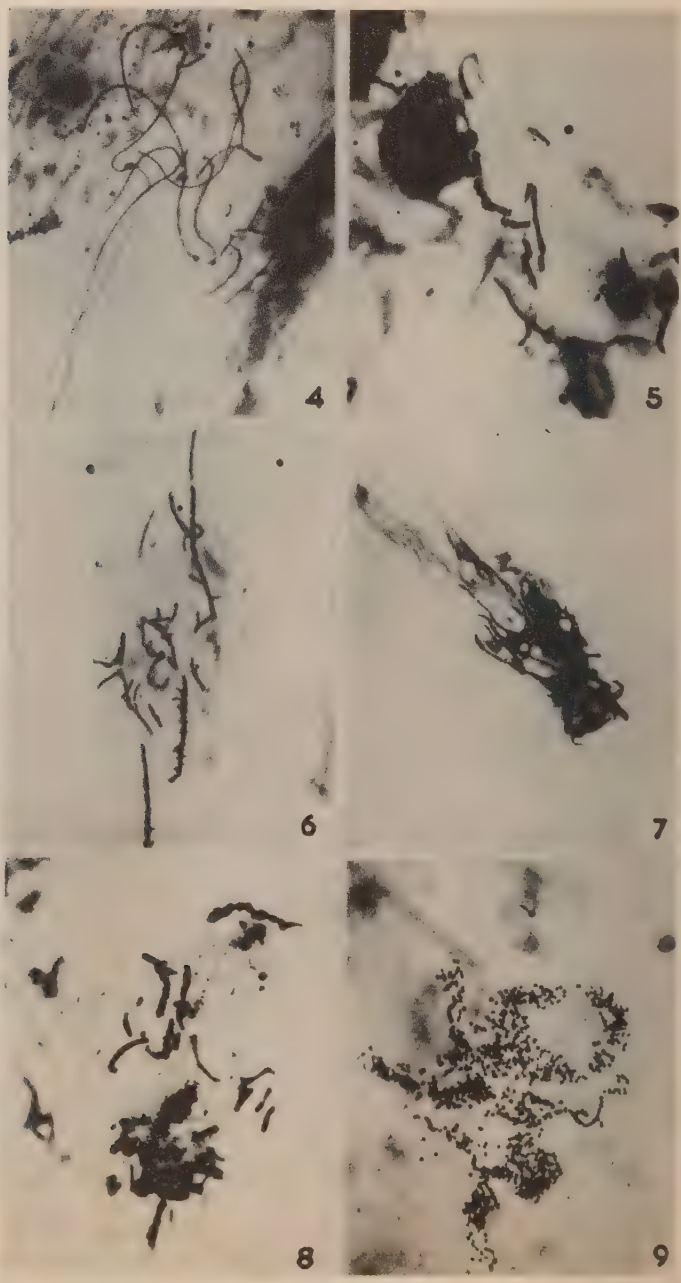
Figs. 5, 8 and 9– x 785

FIG 1

FIG 2

FIG 3

To face page 186


4
5
6
7
8
9

BACTERIAL FLORA OF THE GENITAL TRACT OF EWES UNDERGOING CAESARIAN SECTION

By

Joan B. Hopwood

University of Bristol Field Station, Langford

INTRODUCTION

In an attempt to evaluate the significance of bacterial infection of ewes after caesarian section the flora of the vagina, uterus and peritoneal cavity of 43 ewes was investigated. These animals had undergone caesarian section because of inability to lamb at full term or in an attempt to cure pregnancy toxaemia. The bacteriological records for 14 of these ewes were incomplete. It was hoped to correlate the type of infection present at operation with death or recovery of the ewe.

It seemed likely that many factors other than infection might influence post-operative sequelae. These include the cause of dystocia, the time elapsing between the onset of lambing and operation, the assistance given to the ewe before operation and her physical condition. Records of these factors were therefore made as fully as possible for each ewe. In addition, details of the breed and age of the ewes, and the number, sex and condition of the lambs were noted.

METHODS

Method of Operation

Anaesthesia. The left flank was anaesthetised by blocking the last thoracic and first three lumbar nerves, where they lie between the transverse processes of the lumbar vertebrae using a 2 per cent. solution of procaine hydrochloride.

Technique of operation. The sheep was restrained in the right lateral recumbent position. The left flank was clipped and washed with soap and water followed by cetavlon and surgical spirit. A vertical incision was made about 8in. long beginning approximately 2in. below the tips of the lumbar transverse processes and 2in. behind the last rib. The uterus was exteriorised as much as possible and an incision about 6 in. long made over the lamb's hindquarters or head, avoiding the cotyledons. This lamb was then withdrawn from the uterus and a second or third lamb, if present, was removed through the same incision. The uterus was repaired using catgut and a continuous Lembert suture. The abdominal wall was closed by interrupted mattress sutures of nylon, each suture passing through skin, muscle and peritoneum.

The operation lasted about thirty minutes and the ewe could mother her lambs immediately after it.

After-treatment of Ewes

As a routine procedure each ewe was given 500,000 units of crystalline sodium penicillin G intramuscularly at operation and on each of the following 3 days. The first few cases operated on also received 1.5 to 2.0

ml. of posterior pituitary extract at operation to assist uterine involution, but this was later discontinued. Ewes that appeared to be heavily infected were given more intensive antibiotic therapy. Up to 5 mega units of sodium penicillin G and varying doses of streptomycin sulphate were injected at operation and on each of the following 4 days. A few cases were given 5 ml of concentrated lamb dysentery serum (Burroughs Wellcome). Ewes with a vaginal discharge or retained placenta were treated with streptomycin or mixed streptomycin and penicillin pessaries if the cervix was sufficiently dilated.

Post mortem examinations were made on the 13 ewes which died after operation and on 1 which was destroyed while suffering from spastic paralysis of the hind limbs.

Cultural Methods

Table I shows the number of ewes of each breed which were operated on and the causes of dystocia.

TABLE 1

CAUSES OF DYSTOCIA IN EWES UNDERGOING CAESARIAN SECTION

Breed	*No. of ewes*	*Causes of dystocia*				
		Ringworm (partial dilatation of the cervix)	*Prolapse of vagina*	*Uterine inertia*	*Pregnancy toxaemia*	*Foetal mal-present or oversize*
Kerry Hill	13	8	3	—	1	1
Kerry Hill cross	4	3	—	—	—	1
Clun Forest	7	5	—	1	—	1
Devon Longwool	1	—	—	—	—	1
Devon Closewool	2	1	—	—	—	1
Dorset Down	2	—	—	—	—	2
Southdown	4	2	1	—	—	1
Down (breed not specified)	1	1	—	—	—	—
Ryeland Down cross	1	—	1	—	—	—
Ryeland	2	—	1	—	—	1
Suffolk	3	1	2	—	—	—
Cheviot	1	—	—	—	1	—
Cheviot Border Leicester cross	2	—	1	—	1	—
Total	43	21	9	1	3	9

At the time of operation swabs were taken from the vagina and from the uterus and peritoneal cavity. In the vaginal region the swabs were inserted 8 to 10 cm. where possible, avoiding contamination from the vulva but in cases of vaginal prolapse the cleanest area of the protruding mass was swabbed. In a few cases swabs were taken from the skin, before and after preparing it by washing with soap and water followed by cetavlon and surgical spirit. This procedure was soon discontinued as the cultures were invariably overgrown by *Bacillus subtilis*, probably originating from the air of the operating room which contained hay and straw.

Each swab was cultured at 37°C. on 5 per cent. ox blood agar plates aerobically and anaerobically, and in meat piece broth. If good growth had occurred after 16 to 24 hours separate colonies were picked on to

fresh blood plates. If growth was poor the plates were reincubated for 24 hours before attempting to separate the colonies. The meat piece broth was plated on to 5 per cent. ox blood agar, incubated anaerobically, after heating for 20 minutes at 65°C. if growth was luxuriant, and without heating if it was poor.

Identification of bacteria

Streptococci were identified by their morphology and their colonial appearance on 5 per cent. ox blood agar incubated aerobically for 24 hours.

An attempt was made to classify them by their reactions in peptone water containing 1 per cent. of various sugars and 10 per cent. serum, in methylene blue and litmus milk; by the presence or absence of growth at 45°C. and 10°C. on blood agar after 24 hours; and by the colonial appearance on Edward's medium. Most strains examined did not, however, fall into any well-defined group. Staphylococci were identified by their morphology and colonial appearance. Eleven of 25 strains were white and non-haemolytic; 10 were *a*-haemolytic and 4 β-haemolytic. Small transparent non-haemolytic colonies consisting of cocci arranged in clumps and chains which stained variably by Gram's method and included both aerobic and anaerobic strains are referred to as "small non-haemolytic cocci" (Table 2).

Diphtheroid bacilli were identified by their morphology and by their colonial characteristics.

Coliform bacilli were recognised by their characteristic large, smooth, greyish-white colonies on 5 per cent. ox blood agar incubated aerobically, by the fermentation of lactose and by the production of indole.

Clostridium welchii was identified by the usual biochemical reactions. An attempt was made to classify 13 strains by their toxin production but unfortunately they had to be stored for 2 to 4 months in meat piece broth at 4°C. before testing and 7 died out. The remaining 6 failed to produce toxin in plain meat piece broth made from synthetic and fresh beef, and after enrichment with 1 per cent. glucose. Other media such as those recommended by Keppie and Robertson (1944) and Oakley and Warrack (1953) were not available for trial.

Unidentified Clostridia were organisms which appeared in subcultures from meat piece broth heated to 65°C. for 20 minutes. They were mostly slender rods with oval terminal, or subterminal spores. They were stained variably by Gram's method and could not be grouped by their fermentation reactions. Colonies were spreading, β-haemolytic in some cases, small, irregular and *a*-haemolytic in others.

RESULTS

Table 2 shows the number of cases of each kind of dystocia, the range of bacterial types isolated from the vagina, uterus and peritoneal cavity at operation with the frequency of recovery of each type, and the post-operative sequelae. It is based on 32 cases; of these, eleven died and one was destroyed. None died directly as a result of operation, though shock may have hastened death in three ewes which appeared moribund when admitted and survived for less than 20 hours. One had a jagged tear in the uterine wall, another a severe septic peritonitis and the third died 18 hours after operation

with a torn cervix and haemorrhage into the pelvic cavity. The three ewes suffering from pregnancy toxaemia died 2, 7 and 10 days after operation respectively.

TABLE 2

THE FREQUENCY OF ISOLATION OF DIFFERENT BACTERIA AT OPERATION

Reason for operation	*No. of cases*	*Types of bacteria isolated at time of operation*	*Vagina No. of cases*	*Uterus No. of cases*	*Peritoneal cavity No. of cases*	*Post-operative sequelae*
Pregnancy toxaemia	3	Coliform bacilli	3	—	—	All died
		Staphylococci	3	—	—	
		Small non-haem coccus	1	—	—	
		Diphtheroids	2	—	—	
Ringwomb	14	Coliform bacilli	13	11	1	4 died
		Staphylococci	8	6	—	1 destroyed
		Streptococci	11	8	1	
		Small non-haem. coccus	2	2	—	
		Diphtheroids	8	1	—	
		Cl. welchii	7	10	—	
		Unident. Clostridia	2	2	—	
		C. pyogenes	2	—	—	
Prolapse of vagina	7	Coliform bacilli	7	2	1	3 died
		Staphylococci	2	—	—	
		Streptococci	3	2	1	
		Small non-haem. cocci	2	—	—	
		Diphtheroids	3	—	2	
		Cl. welchii	2	1	1	
		Unident. Clostridia	1	—	—	
Foetal malpresent	5	Coliform bacilli	4	3	2	1 died
		Staphylococci	1	1	1	
		Streptococci	2	—	1	
		Small non-haem. coccus	1	1	—	
		Diphtheroids	2	1	—	
		Cl. welchii	1	1	1	
		Unident. Clostridia	1	1	1	
Foetal oversize Dead lamb in utero Uterine inertia	3	Coliform bacilli	1	2	—	All lived
		Staphylococci	1	—	—	
		Streptococci	2	2	—	
		Small non-haem. coccus	—	1	—	
		Diphtheroids	3	1	—	
		Cl. welchii	2	2	—	

Cl. welchii was isolated from six ewes which died, two in less than 12 hours, and from the one which was destroyed. On the other hand, eight ewes infected by *Cl. welchii* and six out of seven infected by unidentified Clostridia survived. The seventh was destroyed while suffering from spastic paralysis of the hind limbs. Thus the unidentified Clostridia and the strains of *Cl. welchii* isolated appear to have no special pathogenic significance.

The number of ewes suffering from each type of dystocia and the mortalities based on forty-three cases are summarised in Table 3: eleven of these cases were not included in Table 2 as the

TABLE 3

MORTALITY RATES GROUPED ACCORDING TO THE CAUSES OF DYSTOCIA

Indication for caesarian section	*No. of ewes operated on*	*No. of fatal cases*
Ringwomb	21	7
Prolapse of vagina	9	3
Pregnancy toxaemia	3	3
Foetal malpresentation Uterine inertia Foetal oversize	10	1

bacteriological records were incomplete. The total mortality was fourteen. Apart from the cases of ringwomb the numbers are unfortunately too small to be significant.

Table 4 summarises the post-mortem findings for the thirteen ewes which died after operation (26 not included). Five (10, 18, 19, 23 and 30) had retained foetal membranes. In ten (18, 19, 30, 22, 10, 23, 16, 28, 29 and Q2) involution of the uterus had not occurred or was poor and in seven (7, 28, 18, 19, 22, 30 and 31) the uterine incision showed no signs of healing. A septic peritonitis was present in five (16, 19, 28, 29 and 30). *C. pyogenes* was isolated from the peritoneal cavity of three of these cases and from two in which there was no obvious peritonitis. Four cases with severe peritonitis at post mortem had had sterile peritoneal cavities at operation, infection probably having occurred through the uterine incision.

An attempt was made to correlate death or survival of the ewe with the condition of the ewe and the condition of the lambs; the results are summarised in Table 5 which is based on thirty-six cases. Evaluation of the condition of the ewes was inevitably rather arbitrary, but the table does suggest that it might be a useful guide in prognosis.

DISCUSSION

There have been several reports of caesarian section in the ewe during the last fourteen years.

Farquharson (1941) described a successful operation on a 3-year-old Hampshire ewe suffering from uterine torsion. Wright (1950) reported 3 cases, all of which died. In 2 the dystocia was due to insufficient dilatation of the cervix. One of these died from shock and the other from peritonitis. The third was a case of pregnancy toxaemia which died of peritonitis 7 days after operation. No bacteriological examinations were made. Wright suggested that caesarian section might be beneficial in treating pregnancy toxaemia, but this has not been corroborated by other veterinary surgeons and

TABLE 4

POST MORTEM FINDINGS AND BACTERIA ISOLATED FROM 13 EWES WHICH DIED AFTER OPERATION

Ewe No.	*Reason for operation*	*Post-operative survival time*	*Peritonitis*	*Healing of uterine incision*	*Involution of uterus*	*Foetal membranes*	*Condition of endometrium*	*Condition of cervix*	*Bacteria isolated at post mortem*	
									Uterus	*Peritoneal cavity*
10	Pregnancy toxaemia	2 days	—		—	Present	Congested septic contents		Coliform bacilli Streptococcus	Coliform bacilli Coccus *C. pyogenes*
23	Pregnancy toxaemia	7 days	Local	±	±	Present	Severe congestion		Coliform bacilli Staphylococcus	
31	Pregnancy toxaemia	10 days	—	—	+	Absent	Greyish-cream purulent contents	Normal	*Cl. welchii*	Coliform bacilli *C. pyogenes* *Cl. welchii* Unident. Clostridia
16	Ringwomb	2 days	Severe purulent		—		Severe purul. endo-metritis		Coliform bacilli *Cl. welchii* Coccus	Coliform bacilli *C. pyogenes* *Cl. welchii* Coccus
7	Ringwomb	1 day	Fibrinous	—			Severe congestion		Coliform bacilli *Cl. welchii*	
28	Ringwomb	8 hours	Severe purulent	—	—	Absent	Severe congestion		Coliform bacilli Coccus	Coliform bacilli
29	Ringwomb	4 days	Local purulent	±	—	Absent	Partially ruptured. Necrotic cotyledons	Severely bruised. Necrotic mucosa	Coliform bacilli *Cl. welchii* Unident. Clostridia	Coliform bacilli Unident. Clostridia

Q2	Ringwomb	2 days	Slight	±	—	Absent	Severe congestion	Severely bruised		
F2	Ringwomb	5 days	Mild	+		Absent	Necrotic cotyledons	Severely bruised. Necrotic mucosa	Coliform bacilli Staphylococcus Unident. Clostridia	Streptoccoccus Staphylococcus
18	Prolapse of vagina	1 day		—	—	Present	Congested	Partly ruptured		
19	Prolapse of vagina	7 days	Moderately severe purulent	—	±	Present	Congested and oedema-tous		Coliform bacilli *C. pyogenes*	Coliform bacilli *C. pyogenes* Staphylococcus Diphtheroids
30	Prolapse of vagina	5 days	Severe purulent	—	±	Present	Severe congestion	Normal	Coliform bacilli *C. pyogenes* *Cl. welchii*	Coliform bacilli *C. pyogenes* *Cl. welchii*
22	Foetal malpresentation	4 hours	—	—	—	Absent	Severe congestion			

— — absent
± — poor
+ — good

Walsby (1952) stated that the prognosis was very unfavourable in this condition.

Milne (1951) described 5 cases, the indications for operation being (1) pregnancy toxaemia, (2) spasm of the cervix, (3) emphysematous foetus, (4) schistosomus reflexus with uterine rupture, (5) gross oversize of the foetus. Cases (3) and (5) lived in spite of severe bruising of the vagina and purulent endometritis in case (3). No bacteriological studies were made.

TABLE 5

ANALYSIS OF POST-OPERATIVE SEQUELAE

Condition of ewe	*No. of ewes which died*	*No of ewes surviving*
Good	5	13
Fair	—	3
Poor	3	—
Unknown	3	9
Condition of lambs		
Alive	3	14
Dead	6	9
Dead, emphysematous	2	1
Unknown	—	1

Eleven caesarian sections on cases of dystocia due to partial dilatation of the cervix were reported by Harmer and Mount (1951). Two ewes died shortly after operation from "pre-existing septicaemia" and "metroperitonitis" but the bacteria responsible were not determined. A successful operation to relieve foetal malpresentation was described by Arthur (1952). Parkinson (1952) presented case reports of 3 operations in which recoveries were uneventful. The indications for operation were partial dilatation of the cervix, foetal malpresentation and foetal oversize.

The longest report was given by Walsby (1952) on 26 cases of which 7 died. Fatalities included 3 cases of partial dilatation of the cervix associated with "metroperitonitis" two cases of pregnancy toxaemia, one ewe with uterine oedema which died of peritonitis due to failure of the uterus to heal and one ewe which was destroyed at operation because of gross oedema of the uterine wall. Walsby suggests that survival of the ewe depends largely on the prompt expulsion of the foetal membranes and states that if the amnion has been ruptured for over 24 hours before operation the ewe generally dies. Uncomplicated cases of prolapse and of partial dilatation of the cervix usually recover if they are operated on early. Again no bacteriological examinations were reported.

In the above reports no mention is made of any study of the bacterial flora of the uterus or peritoneum. It was the object of this study to try to obtain some information on these points. Partial or complete records for 34 ewes were kept and of these 13 died. All the fatal cases had a severe endometritis associated with a variety of

bacterial species. The uteri varied greatly in gross pathological appearance so that it was not possible to ascertain the casual organisms merely by inspection of the endometrium.

Coliform bacilli were isolated in mixed culture from 9 out of 10 uteri examined post mortem, and at operation from the uteri of 11 cases which lived. Unfortunately the pathogenicity of the strains was not determined and a study of this might prove interesting in future work. *Cl. welchii* was isolated from the uterus in 5 cases at post mortem, but was also obtained at operation from 7 cases which survived. *Corynebact. pyogenes* was isolated from 2 cases of endometritis. It was probably present in others but was overgrown on the blood plates by more luxuriant species.

Peritonitis was present at death in 9 of the 13 ewes and was severe in 6 of them. Three of the 5 cases of purulent peritonitis yielded *Corynebact. pyogenes* in mixed culture, and as the pus produced by this species is extremely resistant to antibiotic penetration, it is potentially a very serious pathogen. *Cl. welchii* was isolated from the peritoneal cavity in 3 cases and coliform bacilli in 7. Five others were not examined bacteriologically and one other yielded only β-haemolytic streptococci and wide-zone-β-haemolytic staphylococci.

Apart from the reports of Harmer and Mount (1951) and of Walsby (1952) it is impossible to compare the survival rates in most of the series of operations reported in the literature as the numbers of cases are too small. In cases of incomplete dilatation of the cervix, Harmer and Mount had a survival rate of 8 out of 11 and Walsby saved 10 out of 13 ewes. In the present study the survival rate was only 14 out of 21: it could undoubtedly have been increased if the ewes had been presented for operation earlier after the onset of labour.

Eight cases of "prolapse of the cervix" were operated on successfully by Walsby, while in the present study 3 out of 9 cases of "prolapse of the vagina" died.

The 3 cases of pregnancy toxaemia reported in this paper died. This observation would appear to substantiate the opinion which is widely held that caesarian section is a very unsatisfactory treatment for this condition.

In view of the variety of bacteria isolated from the genital tract and peritoneal cavity, and the isolation of these organisms at operation in some cases which lived as well as at post mortem in others that died, it appears highly likely that other factors also influence post-operative sequelae. The most important are probably trauma to the genital tract, and delay in involution of the uterus with expulsion of the foetal membranes. Both produce devitalised and dead tissue which forms an excellent culture medium for bacteria. Trauma is frequently caused by forcible attempts to deliver lambs when the cervix is incompletely dilated and by unskilled and

vigorous attempts to correct foetal malpresentations. The repair of vaginal and cervical prolapses is inevitably associated with a certain amount of tissue damage.

CONCLUSIONS

In a series of 43 ewes operated on by caesarian section all the 13 cases which died had a severe endometritis. A variety of bacteria was isolated but none could be incriminated as the primary cause of death.

The potentially pathogenic organisms present included *Corynebacterium pyogenes,* coliform bacilli, staphylococci and *Clostridium welchii.*

ACKNOWLEDGMENTS

My grateful thanks are due to the late Professor F. Blakemore for permission to do this work in his department, and to Professor G. R. Cameron, F.R.S. and Professor J. W. Howie who kindly read the manuscript.

I would also like to thank Professor Messervy and members of the Department of Surgery at the University of Bristol Field Station, Langford for their cooperation in taking swabs from the ewes and undertaking the caesarian sections.

REFERENCES

Arthur, G. H. (1952). *Vet Rec.*, **64**, 482.
Farquharson, J. (1941). *J. Amer. vet. med. Ass.*, **99**, 60.
Harmer, J. B., and Mount, E. D. (1951). *Vet. Rec.*, **63**, 457.
Keppie, J., and Robertson, M. (1944). *J. Path. Bact.*, **56**, 124.
Milne, F. J. (1951). *Vet. Rec.*, **63**, 617.
Oakley, C. L., and Warrack, G. H. (1953). *J. Hyg., Camb.* **51**, 102.
Parkinson, J. D. (1952). *Vet. Rec.*, **64**, 819.
Walsby, J. B. (1952). *Ibid.*, 651.
Wright, J. G. (1950). *Veterinary Obstetrics* p. 332. Bailliere, Tindall and Cox; London.

[*Received for publication, December 5th, 1955*]

A SURVEY OF POULTRY DISEASES IN MID-WALES*

By

F. T. W. Jordan

Veterinary Investigation Centre, Ministry of Agriculture; Aberystwyth†

INTRODUCTION

There are some 92 million poultry in Great Britain (Gordon, 1954), a high proportion of them being kept in small flocks on the general farm (Coles, 1950). The products of the industry are worth about 120 million pounds per annum, and losses from disease are heavy, but few farm surveys of morbidity and mortality have been made. The picture of disease incidence has been largely obtained either from annual reports of the laying trials such as the National Egg Laying Trials and the Poultry Production and Progeny Trials, or from autopsies undertaken at routine laboratories in Great Britain (Matheson, 1927; Kerr, 1928; Matheson and Wilson, 1933; Dalling, Warrack and Duncan, 1937; Montgomery and Warrack, 1938; Dudley, Dobson and Gordon, 1941; Campbell, 1954) and in the U.S.A. (Hutt, 1938; Hoffman and Stover, 1942; Bullis and Olson, 1943; Clayton and Godfrey, 1952).

Two surveys dealing particularly with the incidence of tumours have been made in recent years, one by Goss (1940) in America, and the other by Darcel, Lancaster and Gordon (1952) in Britain.

Figures for disease incidence collected at laying trials are unlikely to be representative of flocks kept commercially, since they relate to a selected population kept for a limited time under standard conditions. Material sent for routine laboratory diagnosis is also unlikely to be representative of total mortality, since it is largely selected by the farmer and little is usually known of the population from which the material is derived.

It was therefore decided to undertake a farm survey of poultry mortality in Mid-Wales and to compare the results with those of routine laboratory findings during the same period.

MATERIALS AND METHODS

Survey farms. Between June 1950 and September 1952 all the poultry on 32 non-accredited farms in Mid-Wales were kept under continuous observation for varying periods of time. Only one of the farms was devoted entirely to poultry and, apart from one backyard poultry keeper, the rest were mixed farms. Of the 32 farms there were 8 in Brecon, 9 in Cardigan, 8 in Montgomery and 7 in Radnor.

The farms were kept under continuous observation for periods varying from 6 months (1 farm) to 24 months (15 farms). The total number of farms under observation at a given time varied because they joined and

*This paper represents part of a thesis submitted for the degree of Ph.D. in the University of London, 1954.

†Present address: Dept. Veterinary Preventive Medicine, University of Liverpool.

left the survey at different times. So long as a farm was included in the survey all relevant details of numbers of poultry stock, breeds, ages, movements on and off the farm, culling, deaths, management, feeding and production were obtained by visits to the farm at least once a month. Every bird that died or was killed because of symptoms of disease was sent, together with the history, to the Veterinary Investigation Centre at Aberystwyth. The routine procedure was to carry out an autopsy and to culture from the heart blood, liver and lungs onto blood agar and D.C.A. medium. Bacteriological examination was extended to other organs and other techniques employed when considered necessary. Histological examination was resorted to in determining the nature of occasional doubtful lesions, but no attempt was made to classify histologically the various neoplastic diseases. When considered necessary, material for toxicological examination was sent to the Veterinary Laboratory, Ministry of Agriculture, Weybridge.

Non-survey farms. The material, comprising all poultry carcases received for routine diagnosis at the Veterinary Investigation Centre at Aberystwyth during the period January 1950 to August 1952, was examined in the same way as those from survey farms, except that when a batch of birds of the same age and from the same flock (usually chicks) was received, only a proportion was examined bacteriologically. Most of the material was received direct from farmers.

RESULTS

General Observations on Losses

The poultry population from which carcases were derived was known for survey farms but could not be ascertained for the routine material. The total number of live birds at different ages on survey farms fell steeply from some ten thousand day olds to only 1,590 birds of 2 years and over. This fall was mainly due to the disposal of birds and only to a less extent to deaths. The largest number of poultry on any of the 32 survey farms was 450 adult birds and about 600 chicks; the smallest was a flock of 10 adult birds belonging to a backyard poultry keeper. The average survey farm carried about 100 to 150 adult stock and 100 to 200 chicks and growers. A total of 2,118 fowl carcases from survey farms were examined between June, 1950 and August, 1952, and 2,341 carcases from non-survey flocks between January, 1950 and June 1952.

The total monthly mortality in the two series is shown in Figs. 1 and 2. It will be seen that for the survey flocks (Fig. 1) the total number of deaths from all causes and the total population ran closely parallel, and what might appear at first sight to be striking seasonal changes in mortality may well be mainly reflections of population changes, although the curve expressing mortality as a percentage of total population suggests that slight seasonal changes in mortality may occur independently of population. The increased percentage death rate in the spring of each year may be due to the greater percentage of losses in young birds from chilling and coccidiosis at these times. For the non-survey material the total monthly mortality is seen in Fig. 2 and the spring peak is seen to

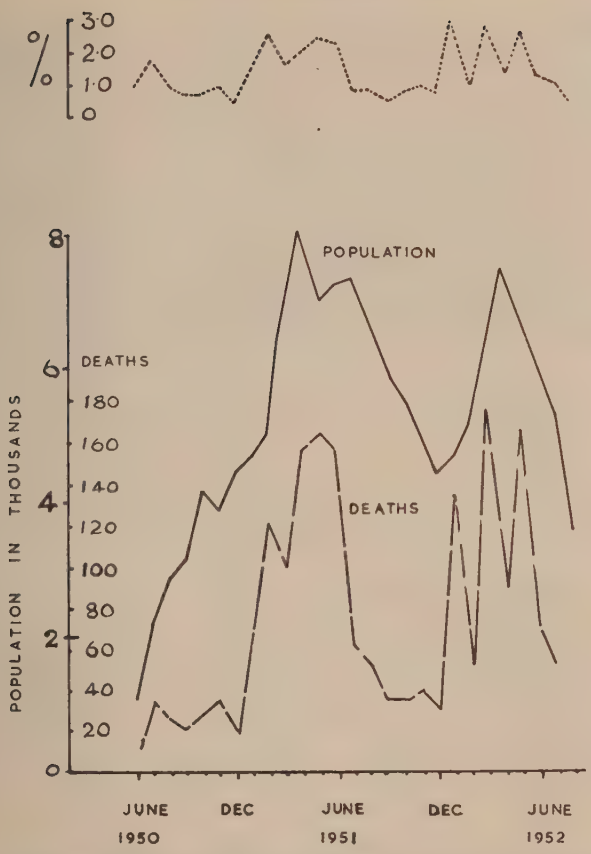


Fig. 1. Total monthly population and deaths from all causes (Survey flocks—all ages). Deaths as percentage of population.

correspond closely with that of the survey flocks (Fig. 1). In each case the losses are analysed for disease among 7 different age groups (Tables 1 and 2).

So far as possible diseases have been classified according to their aetiology; otherwise they have been grouped according to the tissues mainly affected. This method has the effect of greatly reducing the number of cases classified under particular organs and tissues. For instance, although lesions were very common in the digestive system and liver, the classification adopted makes this group a comparatively small one, because most of the lesions in these organs have been classified in other groups e.g. parasitic diseases, tumours.

The most common bacterial diseases encountered in the survey flocks were B.W.D. (76), typhoid (36), tuberculosis (18), staphy-

lococcal infection (18) and coliform infection (2). In the non-survey group they were B.W.D. (96), typhoid (197), *Salm. typhimurium* infection (23), *Salm. thompson* infection (17), tuberculosis (50), staphylococcal infection (15) and coliform infection (5). The parasitic diseases in the survey flocks included:— caecal coccidiosis (228), blackhead (10), *Ascaridia galli* (1) and *S. trachea* (7). In the non-survey flocks they were caecal coccidiosis (229), intestinal coccidiosis (16), blackhead (13), *Ascaridia galli* (2), *S. trachea* (5) red mite (2). Miscellaneous diseases encountered in the survey flocks included coryza (4), poisoning (7), nutritional deficiencies (5) and congenital defects (1). In the non-survey flocks coryza (19), fowl pox (1), omphalitis (6), poisoning (35), nutritional deficiency (105), crooked toe deformity (2) and arthritis of unknown aetiology (13).

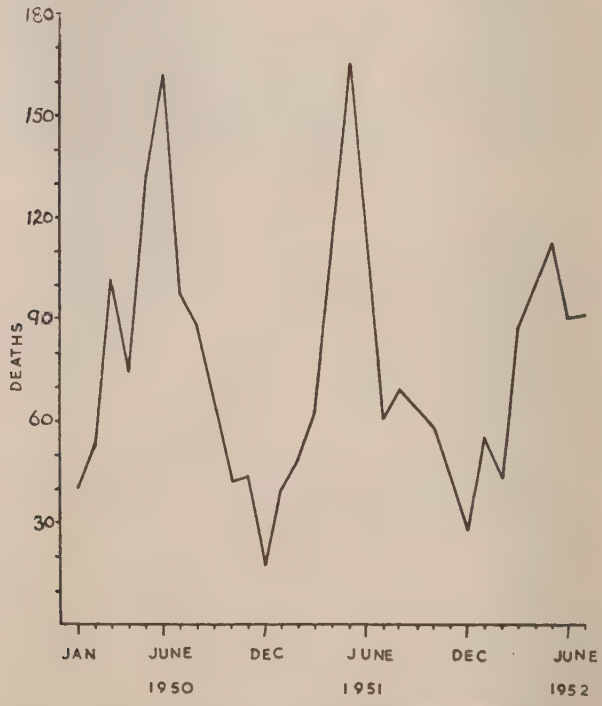


Fig. 2. Total monthly deaths from all causes (Routine diagnosis—birds of all ages).

Deaths in Age Groups

For both the survey and non-survey material, deaths from the various diseases have been expressed as a percentage of total deaths in each of the age groups (Tables 1 and 2). They are also shown graphically (Fig. 3, A and B) but in this case only those diseases which

TABLE I

ANALYSIS OF TOTAL LOSSES IN AGE GROUPS—SURVEY FLOCKS

Diseases	*Chicks*				*Growers*		*Adults*								*Total*	
	0-2 wks.		*2-4 wks.*		*1-6 mths.*		*6-12 mths.*		*12-18 mths.*		*18-24 mths.*		*24 mths. and over*			
	No.	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%
Bacterial Diseases	22	3.7	54	30.0	18	1.9	12	3.7	15	6.6	20	26.5	9	12.3	150	7.0
Aspergillosis	0	0	11	4.9	2	0.3	0	0	0	0	0	0	1	1.4	14	0.7
Parasitic Diseases	0	0	92	51.3	153	23.5	1	0.3	0	0	0	0	0	0	246	11.85
Circulatory	0	0	0	0	2	0.3	3	1.0	4	1.8	2	2.7	1	1.4	12	0.6
Asphyxia	4	0.07	1	0.7	129	19.6	1	0.3	0	0	0	0	0	0	135	6.4
Digestive and Liver	0	0	0	0	23	3.5	13	13.8	8	3.6	3	3.9	3	4.6	50	2.4
Urinary	3	0.05	0	0	4	0.6	9	3.0	6	2.8	2	2.7	3	4.6	27	1.3
Reproductive	0	0	0	0	1	0.2	27	8.8	65	28.5	18	23.7	19	25.7	130	6.3
Tumours	0	0	0	0	73	11.0	166	54.2	72	31.8	10	13.1	19	25.7	340	16.0
Accident and Injury	117	19.5	10	5.6	170	25.6	49	17.0	39	17.9	16	21.2	15	20.2	416	19.5
Chilling	378	64.0	4	2.2	0	0	0	0	0	0	0	0	0	0	382	18.0
Miscellaneous	5	0.08	0	0	8	1.3	3	1.0	0	0	1	1.2	0	0	17	0.75
Undiagnosed	63	10.6	8	4.5	80	12.0	22	6.4	18	7.9	4	5.3	4	5.4	199	9.4
TOTAL	592		180		663		306		227		76		74		2118	100.0

TABLE 2

ANALYSIS OF TOTAL LOSSES IN AGE GROUPS—NON-SURVEY FLOCKS

Disease	*Chicks*				*Growers*		*Adults*								*Total*	
	0-2 wks.		*2-4 wks.*		*1-6 mths.*		*6-12 mths.*		*12-18 mths.*		*18-24 mths.*		*24 mths. and over*			
	No.	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%
Bacterial Diseases	102	25.8	50	21.8	61	8.6	50	10.4	80	22.4	28	34.53	32	32.4	403	16.95
Aspergillosis	5	1.3	2	0.9	3	0.4	3	0.6	2	0.6	1	1.23	0	0	16	0.68
Parasitic Diseases	20	5.05	55	24.0	189	27.0	4	0.8	0	0	0	0	0	0	268	11.39
Circulatory	1	0.25	0	0	8	1.1	7	1.5	13	3.6	2	2.5	6	6.0	37	1.58
Asphyxia	0	0	0	0	26	3.7	0	0	0	0	0	0	2	2.0	28	1.2
Digestive and Liver	0	0	11	4.8	85	12.2	34	7.2	21	5.9	5	6.2	6	6.0	162	6.91
Urinary	0	0	1	0.4	29	4.2	33	6.9	22	6.2	6	7.4	3	3.0	94	3.9
Reproductive	0	0	0	0	3	0.4	50	10.5	91	25.3	21	25.9	24	24.3	189	8.05
Tumours	1	0.25	0	0	114	16.4	212	44.3	82	22.9	13	16.0	15	15.2	437	18.7
Accident and Injury	33	8.4	0	0	13	1.9	6	1.3	9	2.5	0	0	3	3.0	64	2.76
Chilling	105	26.5	12	5.2	0	0	0	0	0	0	0	0	0	0	117	4.98
Miscellaneous	11	2.8	39	17.0	89	12.8	24	5.0	13	3.78	2	2.5	3	3.0	181	8.12
Undiagnosed	117	29.5	60	26.0	79	11.4	56	11.8	25	7.0	3	3.7	5	5.1	345	14.78
TOTAL	395		230		699		479		358		81		99		2341	100.0.

formed 5 per cent or more of total deaths in any age group are shown separately, the others being grouped together as 'miscellaneous' or 'undiagnosed'. The distribution of total deaths between the different age groups was similar for the survey and non-survey groups (Tables 1 and 2).

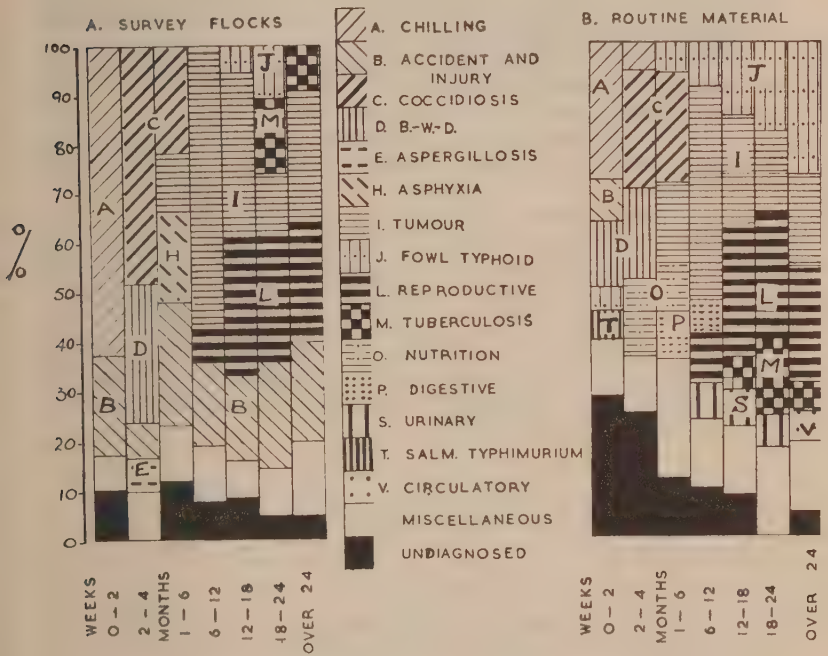


Fig. 3. Analysis of causes of death in age groups.

Chicks (0 to 2 weeks). Over 60 per cent of the losses on survey farms were attributed to chilling, although they were exaggerated by the losses (174) in two successive seasons on one farm as a result of prolonged rail journeys in very cold weather, and the use of inadequately heated brooders. On other farms the losses were much less. Amongst routine carcases 30 per cent of the losses were attributed to chilling. In survey flocks accident and injury caused by vermin, drowning, predatory animals and birds and other means accounted for 20 per cent of all deaths, while in non-survey flocks these conditions were responsible for only 10 per cent of losses. Diseases caused by organisms of the salmonella group were of little importance in survey birds, whereas they were responsible for nearly 30 per cent of losses amongst the non-survey group. Blackhead was diagnosed in a 13 day old chick.

Chicks (*2 to 4 weeks*). Coccidiosis and B.W.D. were the two commonest causes of death in both survey and non-survey series, but whereas they caused nearly 80 per cent of the total losses in the survey group they were responsible for only half that number in the non-survey group. Blackhead occurred in 2 survey chicks in this age group, one being 17 and the other 19 days old. Nutritional deficiencies were responsible for about 12 per cent of the total deaths among the non-survey carcases.

Growers (*1 to 6 months*). Accident and injury, and asphyxia due to crowding together of young birds, caused nearly half of the total deaths amongst survey birds, whereas they accounted for very few of the deaths in the routine series. In both series tumour conditions, including neurolymphomatosis and coccidiosis, were important causes of loss.

Adults (*over 6 months*). In both survey and routine material, tumours and disease of the reproductive system (mainly egg peritonitis) were amongst the most important causes of loss. Accident and injury caused a high proportion of the deaths among survey birds but were uncommon in the routine material. The high incidence of fowl typhoid in the non-survey material was probably due to the fact that since it was known that the author was interested in this disease, poultry keepers who recognised it clinically were more likely to forward carcases.

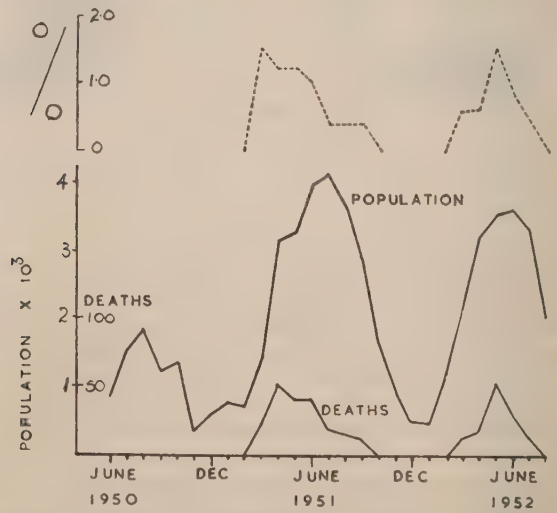


Fig. 4. Monthly population and deaths from coccidiosis (Survey flocks—birds 0-6 months old).
Deaths as a percentage of population.....

The Incidence of Some of the more Important Diseases

Coccidiosis. Caecal coccidiosis was an important cause of death in

both series, but intestinal coccidiosis was seen only amongst routine autopsies and in only 16 cases. In the 0 to 6 months age group the incidence of caecal coccidiosis in birds from survey farms was marked by peaks in April and May (Fig. 4) while the routine material showed three peaks (Fig. 5) at about the same time (May to June). Most of the deaths from caecal coccidiosis occurred in birds of 4 to 6 weeks of age, although one chick from the non-survey group died of the condition when only 5 days old, which means that infection must have been acquired within a few hours of hatching.

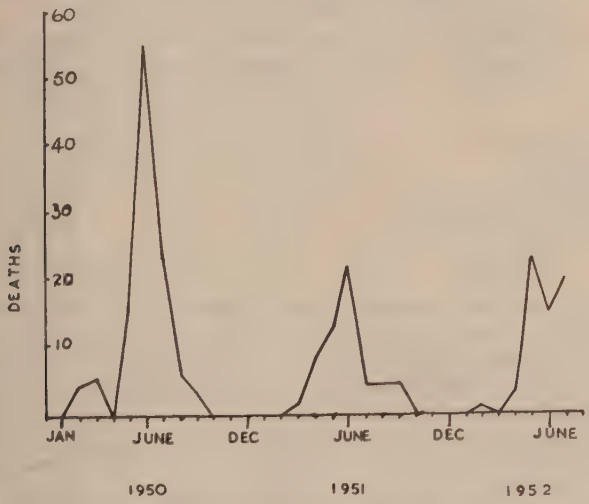


Fig. 5. Monthly deaths from coccidiosis (Routine diagnosis—birds 0-6 months old).

Tumours. Under this heading only those birds are included in which neoplasia was considered to be the cause of death. In nearly every case where tumours were found they were in fact the cause of death or destruction, and therefore the incidence of tumours classified under this heading is only slightly lower than their real incidence.

There is much difference of opinion regarding the classification of neoplasms, and there is also doubt as to whether neurolymphomatosis, the ocular form of the leukosis complex and osteopetrosis are truly neoplastic conditions. For this and other reasons it was decided to classify tumours on the basis of macroscopic examination alone under the following six headings:— neurolymphomatosis, haemangioma, myelocytoma, osteopetrosis, ocular form of the leukosis complex, and 'other neoplasms', most of which were probably the visceral form of the leukosis complex. Although this reliance on macroscopic examination alone may have given rise to error, it was felt that it gave a fair picture of deaths from neoplastic diseases in relation to other diseases and also reflected the relative incidence of

the main macroscopically recognisable groups of neoplastic conditions.

Table 3 shows the number of each type of tumour for both survey and non-survey flocks. Tumours were responsible for 16 per cent of total deaths on survey farms and for 19 per cent in the routine material. It is of interest that in the 6 to 12 months age group, tumours were responsible for about half of all the deaths in both series.

TABLE 3

THE INCIDENCE OF VARIOUS TYPES OF TUMOURS IN MATERIAL FROM SURVEY AND NON-SURVEY FARMS

Type	*Number of tumours*		*Total*
	Survey Farms	*Non-Survey Farms*	
Neurolymphomatosis	52	75	127
Haemangioma	16	16	32
Myelocytoma	5	14	19
Osteopetrosis	0	2	2
Ocular form of Leukosis Complex	0	2	2
'Other neoplasms'	267	328	595
TOTAL ..	340	437	777

Neurolymphomatosis was found chiefly in birds of 4 to 6 months of age, and indeed caused most of the tumour deaths recorded in the 1 to 6 months age group. In this group there were 45 cases of neurolymphomatosis out of a total of 52 cases of tumours in the survey series, and 58 out of 75 in the non-survey material. Haemangiomata were seen affecting the viscera in 26 birds, and the skin in six. Osteopetrosis and the ocular form of lymphomatosis were each seen only twice. Most of the tumours classified as 'other neoplasms' were probably the visceral form of the leukosis complex. They were seen in 595 birds, over half of them (295) being in the 6 to 12 months age group.

The monthly distribution of deaths from tumours in the 1 to 6 and 6 to 12 months age groups shows a similar pattern for survey flocks (Fig. 6) and for routine carcases (Fig. 7). In the younger of the two age groups most losses occurred in the summer months, whilst in the older group most occurred in the winter.

For the survey flocks it was possible in both age groups to give a population curve (Fig. 6) which shows that the incidence of tumours closely followed the population changes with no clear evidence of a seasonal incidence unrelated to population. In the survey flocks details of breed, age, sex, diet, housing and other environmental factors were known, but they were so varied that it was impossible to relate them to the incidence of tumours.

Disease of the reproductive system. The chief condition encountered was egg peritonitis, together with a few cases of prolapsus. In both the survey and non-survey series they were rare in birds under 6

months of age, but caused about 25 per cent of all deaths in each of the older age groups (Tables 1 and 2). In both series two-thirds of all the deaths from reproductive disease were in birds over 12 months old, although less than one-third of all the carcases examined were in this age group. Monthly losses from reproductive disease are shown in Fig. 8 (Survey flocks) and Fig. 9 (Routine diagnosis). In the case o survey flocks, for which the population was known, the incidence follows closely the changes in population, and does not appear to be influenced by season.

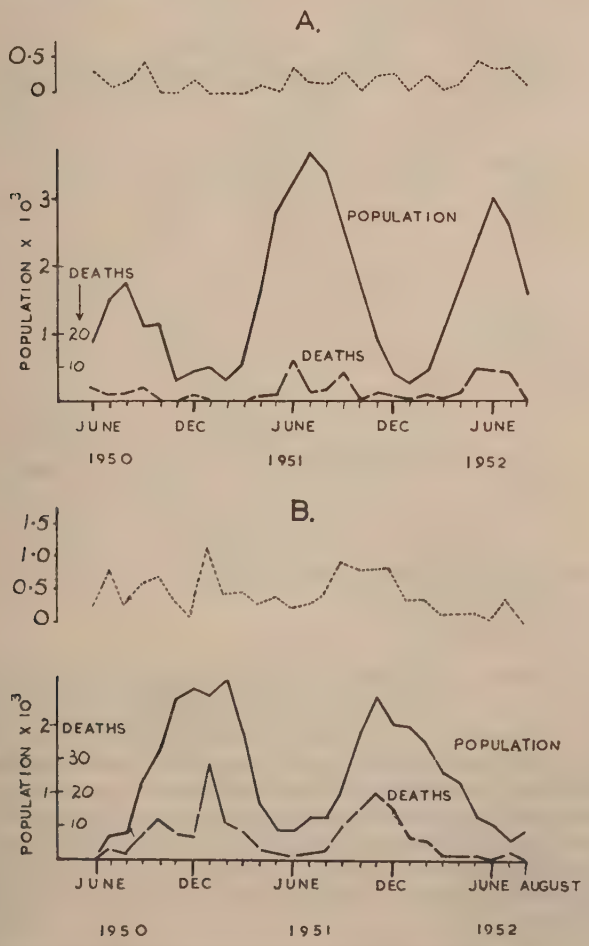


Fig. 6. Monthly population and deaths from tumours (Survey flocks).
A. Birds 1-6 months
B. Birds 6-12 months
Deaths as a percentage of population.....

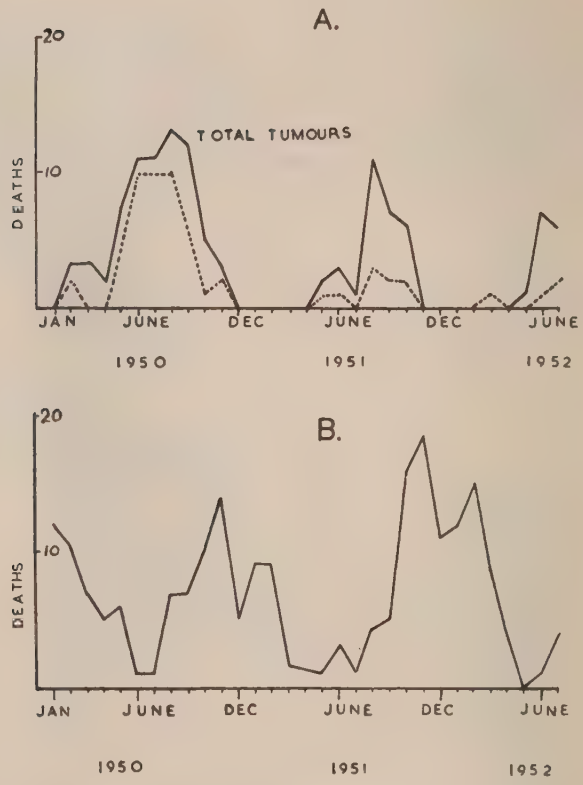


Fig. 7. Monthly deaths from tumours (Routine diagnosis).
A. Birds 1-6 months
B. Birds 6-12 months.
Neurolymphomatosis

Diseases of the urinary system. The conditions encountered included nephritis (with or without excessive deposition of renal urates), visceral gout and inflammation of the ureter. All the cases of nephritis were sporadic with no evidence of outbreaks suggestive of pullet disease. Deaths occurred in all age groups.

Nutritional deficiencies. The only condition encountered in the survey flocks was riboflavin deficiency (5). In the routine material there were instances of rickets (47), crazy chick disease (44), vitamin A deficiency (2), riboflavin deficiency (3), perosis (2) and chick dermatitis (7).

Accident and injury. Although this group of diseases was comparatively insignificant as a cause of death in the routine material, it was the most important single cause in the survey flocks where it

was of importance in all age groups. It included death by drowning, hover fires, trampling of chicks, and injury and attack by predatory animals and birds.

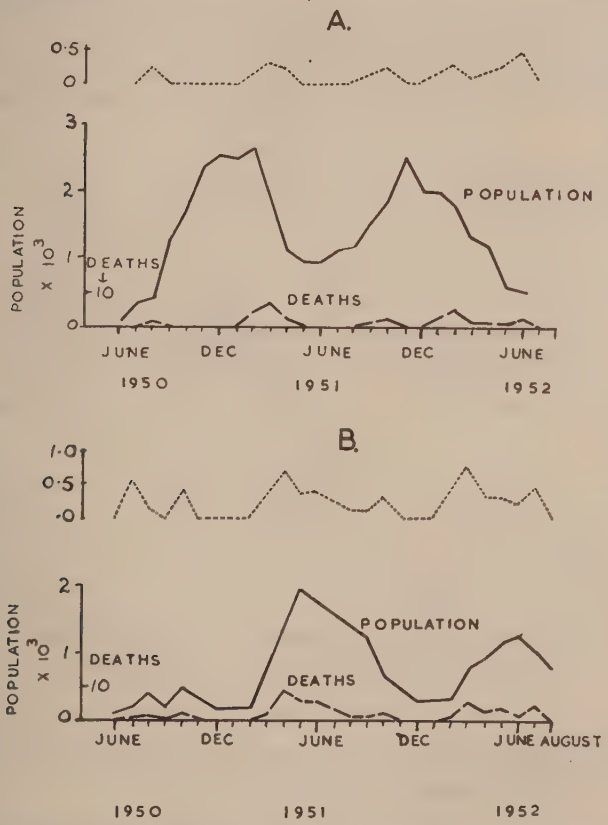


Fig. 8. Monthly population and deaths from reproductive disease (Survey flocks).
A. Birds 6-12 months
B. Birds 12-18 months
Deaths as a percentage of population.....

Undiagnosed cases. The designation 'undiagnosed' was used when a study of the history, post-mortem findings, bacteriological examination and in some cases chemical analysis failed to yield a diagnosis. Post-mortem decomposition precluded diagnosis in a small number of cases. Undiagnosed cases comprised about 10 per cent of all carcases from survey farms and about 15 per cent of the routine material.

General

At the outset of the farm survey it had been hoped to correlate the incidence of disease with such factors as breed, management,

feeding, hygiene and locality. It was realised, however, as the work progressed, that the great variations in these factors made this impossible.

All the common diseases were found in each of the 4 counties, and their incidence did not differ greatly from one county to another. Examination of a much larger population, however, might well show that certain diseases, such as fowl typhoid, are more common in certain areas.

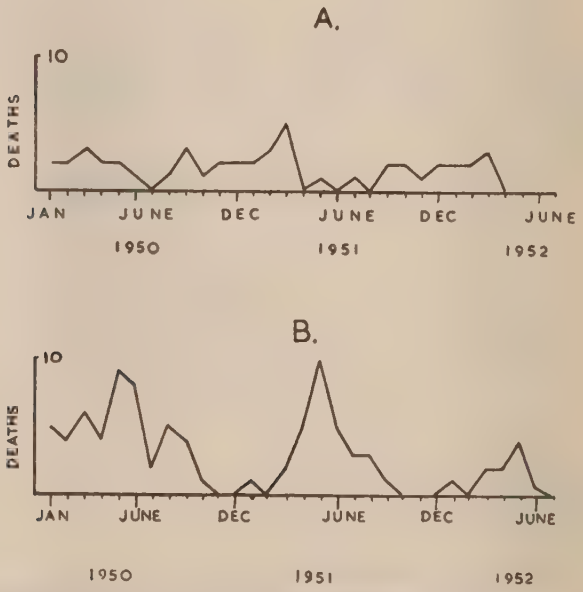


Fig. 9. Monthly deaths from reproductive disease (Routine diagnosis).
A. Birds 6-12 months
B. Birds 12-18 months

Because of the continuous traffic in poultry on the survey farms, and changes in the number of farms on the survey from month to month, there were very few flocks in which the birds were under observation for the whole period of 24 months. A number of flocks, however, were under continuous observation for at least one or more age groups (Table 4), and these flocks provide information about total mortality in relation to the population at risk in each age group. This population ranged from some 10,000 chicks between day old and two weeks to only some 300 birds between 18 and 24 months. Deaths accounted for only a proportion of the population differences in the successive age groups. The main cause of this fall in population was disposal of birds for other reasons. The last column in Table 4 is the percentage loss in each age group, calculated as if the same flock had been under observation from the first day until 2 years of age

and no birds had been disposed of except those that died. By adding together the percentage losses, in this column, it can be seen that the total mortality over the first 24 months would then be 26.9 per cent. Tables 5 and 6 show the four diseases which accounted for most deaths in chicks (0 to 4 weeks), growers (1 to 6 months), and adults (6 months and over) in survey and routine material respectively.

TABLE 4

LOSSES IN BIRDS OF DIFFERENT AGE GROUPS RELATIVE TO POPULATION (SURVEY FLOCKS)

Age and period of observation	*Number of birds*	*Deaths*		*Corrected death rate*
		Number	%	
0-2 weeks	10,275	578	5·5	5·5
2-4 ,,	9,002	175	2.0	1.8
1-6 months	3,431	232	6.8	5·9
6-12 ,,	1,275	71	5·7	4·8
12-18 ,,	637	51	8.0	6.6
18-24 ,,	320	10	3.0	2.3
0-24 ,,	—	—	—	26.9

TABLE 5

THE COMMONEST CAUSES OF DEATHS IN CHICKS, GROWERS AND ADULTS (SURVEY FLOCKS)

Age group	*Number of deaths*				
	All causes	*Commonest causes*			
		(1)	(2)	(3)	(4)
Chicks		Chilling	Accident and Injury	Coccidiosis	B.W.D.
(0-4 weeks)	772	382 (50%)	127 (13%)	87 (11%)	76 (10%)
Growers		Accident and Injury	Coccidiosis	Asphyxia	Tumours
(1-6 months)	663	370 (26%)	141 (21%)	129 (20%)	73 (11%)
Adults		Tumours	Reproductive	Accident and Injury	Typhoid
(over 6 months)	683	267 (39%)	129 (19%)	119 (18%)	33 (5%)
All ages		Accident and Injury	Chilling	Tumours	Coccidiosis
	2118	416 (20%)	382 (18%)	340 (16.5%)	228 (11%)

DISCUSSION

It is of interest to compare the results obtained with material from survey and non-survey farms with each other and with the results obtained by other workers. The small proportion of total poultry deaths which a routine laboratory may receive is shown by a comparison of the total carcases forwarded from survey farms and the carcases received under routine conditions. The former were

derived from a poultry population which varied from some 10,675 day old chicks to 1,500 birds at 24 months of age. During a slightly longer period, a total of only 2,341 carcases were received from the rest of Mid-Wales, comprising many hundreds of flocks and representing at that time a poultry population of some one and a half million birds.

TABLE 6

THE COMMONEST CAUSES OF DEATH IN CHICKS, GROWERS AND ADULTS (ROUTINE DIAGNOSIS)

Age group	*Number of deaths*				
	All causes	*Commonest causes*			
Chicks (0-4 weeks)	625	Chilling 117 (19%)	B.W.D. 91 (15%)	Coccidiosis 74 (12%)	Nutritional 41 (7%)
Growers (1-6 months)	699	Caecal coccidiosis 155 (22%)	Tumours 114 (16%)	Digestive 80 (11%)	Nutritional 59 (8%)
Adults over 6 months	1017	Tumours 322 (32%)	Reproductive 185 (19%)	Typhoid 133 (13%)	Urinary 64 (6%)
All age groups	2341	Tumours 437 (19%)	Caecal coccidiosis 229 (10%)	Typhoid 197 (8%)	Reproductive 189 (8%)

Over the first 18 months of life the total poultry mortality in survey flocks expresed as a percentage of the population was nearly 25 per cent which is comparable with the range of 18.8 to 24.6 per cent quoted by Gordon (1954) for deaths occurring from day old up to the end of the first laying season at the Poultry Production and Progeny Trials from 1949 to 1953. Gordon's findings, however, do not include losses due to infectious diseases, chilling, or accident and injury. Over the production period (6 to 18 months) the mortality in the survey was 11.4 per cent which is similar to the figures quoted by Gordon (1954) of 9.4 to 9.85 per cent for the National Laying Test 1949-1953 and 10.1 to 14.58 per cent for the Poultry Production and Progeny Trials over the same period.

Accident and injury was the commonest single cause of death on survey farms, and was among the three most important causes of death in chicks, growers and adults. These conditions are of little significance in laying and production trials, probably because of the very high standard of management and the protection from predators afforded to the poultry. This high standard is not attained on the general farm and many deaths from these conditions might therefore at first sight be expected in a routine laboratory survey. That this is not so can be seen from the small proportion of deaths attributed to this cause amongst our routine cases, and this is undoubtedly due to the fact that farmers readily recognise such causes of death and are

thus unlikely to submit carcases for routine laboratory examination. The same remarks apply also to asphyxia, which is a frequent sequel to birds crowding into a corner of a poultry house.

Deaths from the more rapidly infectious diseases like coccidiosis and salmonella infections are infrequently seen in laying and production trials, because of the high standard of management and the precautions taken with entrants. These diseases are, however, important causes of death on the general farm, and this is reflected in the material received for routine examination and from survey farms. In the case of the former, however, only a sample of birds dying of these diseases are selected by the owner and sent for examination, whereas in the case of survey farms all carcases were obtained and a more accurate assessment of the significance of these diseases could be made. In the present work this is illustrated by comparing the proportion of total deaths due to B.W.D. and coccidiosis for survey and routine material in the 2 to 4 week age group (Figs. 3A and B).

During the months of the year when coccidiosis caused deaths on survey farms, its incidence appeared to reflect the population change (Fig. 4), peak deaths closely corresponding to, but slightly preceding the population peak of birds in the 0 to 6 months age group. Mortality was highest in April and May, at a time when 4 to 6 week old birds (the most susceptible age) were most numerous. It is not known, however, whether the increased percentage mortality in March of each year is due to increased numbers of susceptible birds favouring the spread of the disease or whether it represents a true seasonal rise unrelated to population.

Undiagnosed cases may comprise 15 to 20 per cent of total deaths in routine laboratories. The comparatively low figure for the survey flocks was probably due to the complete histories forwarded with each carcase and the knowledge of each farm from which they were derived. An example is chilling, for which there are no pathognomonic lesions and in which diagnosis is dependent upon an accurate history. Many routine cases without such histories are inevitably classified in the undiagnosed group.

Much of the published work on the incidence of avian tumours has been undertaken on material received at a laboratory for autopsy, sometimes with no accurate knowledge of the age or population of the birds from which the material was obtained. In the survey reported here the proportion of deaths in birds of all ages attributed to tumours was 16.5 per cent and in routine material 18.7 per cent. These figures are in close agreement with the figure of 17 per cent given by Campbell (1945) for routine post mortem material from 2,063 birds. Many other workers over the years have quoted the incidence of tumours in different age groups, the figures varying from only 0.17 per cent to as high as 19.5 per cent but these figures relate to total incidence, and not to deaths actually caused by tumours. According to Engelbreth-Holm (1942), numerous workers have suggested that the peak incidence for spontaneous tumours

occurs in winter and spring. Campbell, (1945), in an analysis of 2,063 cases of natural neoplasia, during 1939 to 1944, suggests the occurrence of a spring peak associated with increased endocrine activity in that season, but in a later paper (Campbell, 1954) when, analysing a larger number of autopsies (3,285 examined during 1939 to 1947), he found the peak incidence in October and attributed this mainly to neurolymphomatosis. As has already been pointed out for survey farms, the monthly incidence of tumours in the two age groups 1 to 6 and 6 to 12 months follows closely the population incidence, with no clear evidence of a seasonal incidence unrelated to population (Fig. 6).

In view of the suggestion of Wilson (1944) and Darcel, *et al.* (1952) of a possible relationship between coccidiosis and neurolymphomatosis, it is of interest that on the survey farms deaths from caecal coccidiosis occurred on only four of the twelve farms where neurolymphomatosis was recognised, and deaths from duodenal coccidiosis on none of them. Only two cases of osteopetrosis were seen among the survey and routine material, confirming the observation of Darcel, *et al.* (1952) that the condition is rare in this country.

Losses from disease of the reproductive system on survey farms in the 6 to 12 and 12 to 18 months age groups (Fig. 8) closely follow the changes in population, and are not influenced by season. In contrast, Dudley, *et al.* (1941), analysing the autopsies from egg-laying trials (1929 to 1937), found the incidence of reproductive disease, which is mainly egg peritonitis, was higher during the late spring and summer months, and from the frequent occurrence of misshapen ova suggested that degeneration of the ovary due to salmonella infection might be responsible. In our survey and routine series the mortality from reproductive disorders over the period of 6 to 18 months of age was 17 per cent of total deaths. Dudley, *et al.* (1941) found that the incidence over approximately this age group varied from about 40 per cent of the total mortality in 1929 to 1930 to 20 per cent in 1936 to 1937. In the Poultry Production and Progeny Trials over the years 1949 to 1953 (Anon. 1949 to 1953a) reproductive disorders accounted for between 9 and 31 per cent of the total mortality in the production period. During the same years the corresponding average losses at the National Egg Laying Trials (Anon 1949-53b) were about 30 per cent. Reproductive disorders in both surveys rose from about 10 per cent of the total mortality in the 6 to 12 months age group to nearly 30 per cent in the 12 to 18 months age group (Tables 1 and 2), and a similar increase was recorded by Dudley, *et al.* (1941) and Darcel, *et al.* (1952).

Blackhead caused the death of one 9 days old chick in the non-survey group and one 17 days old chick in the survey group, which shows that the incubation period may be even shorter than the 19 days minimum recorded by Stephenson and Hughes (1954) for turkey poults deliberately exposed to infection at a month old.

CONCLUSIONS

A survey of poultry mortality was carried out during 1950 to 1952 on 32 farms in the 4 counties of Mid-Wales. Frequent visits were made to these farms and all birds that died or were killed because of disease, comprising 2,118 carcases, were examined. During the same period a study was also made of the causes of death of a total of 2,341 poultry sent from the 4 counties of Mid-Wales to the Veterinary Investigation Centre at Aberystwyth for routine diagnosis.

The total population of live birds of different ages on the survey farms ranged from nearly 11,000 day old chicks to 1,500 two year old birds. The population of poultry from which the routine carcases were derived was not known.

On the survey farms the most common causes of deaths were accident and injury (20 per cent), chilling (18 per cent), tumours (16.5 per cent), and caecal coccidiosis (11 per cent). Neurolymphomatosis was mainly found in birds 1 to 6 months of age, and was responsible for more than half of the deaths due to tumours in this age group. Egg peritonitis occurred most commonly in birds over 12 months of age. Undiagnosed cases amounted to nearly 10 per cent of total deaths.

Analysis of the monthly deaths from caecal coccidiosis showed that the incidence in the late spring was proportionally higher than the increase in population of susceptible birds at that time of the year. Similar analyses for tumours and reproductive disease showed no clear evidence of seasonal incidence unrelated to population.

Total losses from all causes during the first 18 months and 2 years of life were estimated at 24.6 per cent and 29.6 per cent respectively.

The most common causes of death in the non-survey material were tumours (19 per cent), caecal coccidiosis (10 per cent), fowl typhoid (8 per cent), and reproductive disease (8 per cent). In birds over 6 months old, 32 per cent of all deaths were due to tumours. The cause of death could not be determined in 15 per cent of carcases.

The results of the two surveys are compared to show that there are several factors which limit the value of data obtained from routine laboratories in estimating poultry mortality. On the other hand, the large number of farms served by a routine laboratory is likely to provide a greater variety of diseases.

ACKNOWLEDGMENTS

I wish to thank Professor Lovell of the Department of Pathology of the Royal Veterinary College, London for guidance and advice, and Professor White for help in presentation.

For technical assistance I am especially grateful to Messrs D. G. Davies, W. W. Williams, D. W. Richards and Miss M. M. Evans of the Veterinary Investigation Centre, Ministry of Agriculture, Aberystwyth.

Salmonella cultures were identified by Dr. Joan Taylor of the Salmonella Reference Laboratories, Colindale, London and Mr. J. D. Blaxland of the Veterinary Laboratories of the Ministry of Agriculture,

Weybridge, and yeasts and fungi by Dr. Ainsworth of the University of Exeter.

I would also like to thank the veterinary officers, poultry advisory officers and farmers who co-operated in this work.

REFERENCES

————(1949-53a). *Register of Poultry Production and Progeny Trials.*
————(1949-53b). *Register of National Laying Tests.* 9-12.
Bullis, K. L., and Olson, C. J. (1943). *Amer. J. vet. Res.*, **4**, 382.
Campbell, J. G. (1945). *J. comp. Path.*, **55**, 308; (1954). *Proc. 10th Worlds Poult. Cong.*, 193.
Clayton, C. B., and Godfrey, G. F. (1952). *Poult. Sci.*, **31**, 149.
Coles, R. (1950). *Agriculture*, **56**, 332.
Dalling, T., Warrack, G. H., and Duncan, E. H. L. (1937). *Rep. Reg. Sci. Poult. Breed. Assoc.* 118.
Darcel, C. le Q., Lancaster, J. E., and Gordon, R. F. (1952). *Brit. vet. J.*, **108**, 338.
Dudley, F. J., Dobson, N., and Gordon, R. F. (1941). *Vet. Rec.*, **53**, 323.
Engelbreth-Holm, J. (1942). *Leukaemia in Animals.* Oliver and Boyd; Edinburgh.
Gordon, R. F. (1954). *Vet. Rec.*, **66**, 828.
Goss, L. J. (1940). *Cornell Vet.*, **30**, 75.
Hoffman, H. A., and Stover, D. E. (1942). *Bull. Calif. Dep. Agric.*, **31**, 1.
Hutt, F. B. (1938). *Amer. Nat.*, **72**, 268.
Kerr, W. R. (1928). *Vet. J.*, **84**, 584.
Matheson, D. C. (1927). *Proc. 3rd. World's Poultry Cong.*, 270.
Matheson, D. C., and Wilson, J. E. (1933). *Proc. 5th. World's Poultry Cong.*, 3.
Montgomery, R. F., and Warrack, G. H. (1938). *Rep. Reg. Sci. Poult. Breed. Assoc.*, 24.
Olson, C. Jr., and Bullis, K. L. (1942). *Mass. agric. Exp. Sta. Bull.* 391.
Stephenson, J., and Hughes, D. L. (1954). *Proc. 10th. World's Poultry Cong.*, 282.
Wilson., J. E. (1944). *Vet. Rec.*, **56**, 521.

[*Received for publication, December 6th, 1955*]

THIAMINE DEFICIENCY ENCEPHALOPATHY IN CATS

By

K. V. Jubb*, L. Z. Saunders and Helen V. Coates

N.Y. State Veterinary College, Ithaca, N.Y., and Brookhaven National Laboratory, Upton, N.Y.

INTRODUCTION

Green (1936) described as Chastek paralysis a distinctive clinical syndrome with predominantly nervous manifestations occurring in foxes fed raw carp (*Cyprinus carpio*). Green, Evans and Carlson (1937) reported that the disease could be cured by thiamine, and their experimental reproduction of the disease was confirmed by Ender and Helgebosted (1939). Carlström and Jonsson (1938) reported the disease from Sweden and the recovery of one fox following injection of thiamine. In 1943, Long and Shaw reported Chastek paralysis in mink and foxes in Oregon. The animals had been fed fresh frozen Columbia river smelt (*Thaleichthys pacificus*).

In a paper describing the lesions of Chastek paralysis in foxes, Evans, Carlson and Green (1942) briefly mentioned an experiment in which 25 mg. of thiamine administered daily prevented the occurrence of the disease in foxes fed raw carp as 20 per cent of the ration. These authors described in detail the distribution of the typical vascular lesions in the brains of foxes dying of Chastek paralysis. The remarkable bilateral symmetry and localisation of the lesions in certain of the nuclei of the paraventricular grey matter were compared with the lesions produced by thiamine deficiency in rats (Prickett, 1934), in dogs (Zimmerman, 1939), and in chickens (Pappenheimer and Goettsch, 1931). Their conclusions supported the contention made previously by Alexander (1940) that the diseases so produced were analogous in their pathological features to Wernicke's "haemorrhagic encephalitis" and associated with a deficiency of vitamin B1.

Everett (1944) produced an acute nervous disorder in cats fed canned dog food and brewer's yeast which had been autoclaved to destroy thiamine. The adequacy of the diet in respect to essential nutrients other than thiamine was ensured by the addition of other vitamins and demonstrated by observing the prophylactic and therapeutic efficiency of thiamine. The description and interpretation of the clinical syndrome could not be easily improved; unfortunately, the conclusion that the midbrain was most severely affected was not confirmed histologically.

The clinical syndrome in Everett's cats was divided into three stages. In the stage of induction or non-specific disturbance there was a progressive decline in food consumption, although interest in food was maintained, and vomiting occurred. The critical phase was characterised by a variety of nervous signs, the most constant of which were postural abnormalities, ataxia and pupil dilatation. Convulsions, head ventroflexion and spastic tonus were frequent signs. Within three or four days the cats passed into the terminal irreversible stage of coma and maintained extensor tonus. Smith and Proutt (1944) working in the same laboratory were able to reproduce the clinical disturbances described by Everett by feeding cats a diet of carp or salt water herring (*Merluccius*

*Present address: Hexham, Victoria, Australia.

bilinearis). The onset occurred 23 to 40 days after the cats were placed on the fish diet and in the early stages were reversible by injected thiamine. These two species of fish were among those in which Deutsch and Hasler (1943), in an extensive survey, described the presence of a thiamine-splitting enzyme previously recorded by Woolley (1941), and by Sealock, Livermore and Evans (1943). Cats fed on fish known not to contain the thiaminase (Deutsch and Hasler, 1943) were not affected.

The present report is concerned with a naturally occurring disease of cats characterised by nervous dysfunction similar to that described for the experimentally thiamine deficient cats and by lesions of the central nervous system entirely analogous to those attributable to thiamine deficiency in the other species.

MATERIAL AND METHODS

The study is based on ten naturally occurring cases that were presented to us for diagnosis and treatment, and on four experimentally produced cases. In each case for which the information is available, the disease occurred in cats fed a diet of canned commercial cat food containing whole fish, and it has been reproduced in two cats by feeding the same commercial product as the sole ration. Our presumptive diagnosis of thiamine deficiency in the first case was given support by Dr. P. Olafson (1954), who showed us an identical lesion that had been produced experimentally in foxes some years ago.

In an attempt to determine whether the condition was due to thiamine deficiency two more cats were fed autoclaved canned cat food as the sole ration. Vitamins were given by intraperitoneal injection twice weekly in an amount equivalent to a daily dosage of 0.04 mg. pyridoxine hydrochloride, 1.0 mg. calcium pentothenate, 0.5 mg. riboflavin and 1.0 mg. niacin. The vitamin injections were necessary because the cats developed anorexia on this ration. This essentially repeated Everett's experimental conditions, differing only in the addition of the niacin, which is now known to have neurotrophic effects. These two cats developed a neurological dysfunction similar to that produced in Everett's cats and clinically and pathologically similar to the natural disease. Very rapid improvement from the critical phase followed the intraperitoneal injection of 1 mg. of crystalline thiamine HCl. When maintained on the above diet and killed in extremis in the recurrent phase of nervous dysfunction the cats showed typical brain lesions.

RESULTS

Clinical Signs

This description covers the salient features of the disease. Naturally occurring cases are usually not presented for diagnosis until the later stages of the induction phase or the early critical stage. However, in the two cats fed the commercial diet, weight gains were normal until the eleventh day. Food consumption then decreased until the twenty-second day when anorexia was nearly complete and body weight was reduced by 25 per cent. There was occasional vomiting and excessive salivation. Ataxia was first noted 29 and 31 days respectively after the cats were placed on this diet. In all cats the nervous manifestations of the critical phase were similar to those

described by Everett (1944). The early ataxia, weaving and high limb movements were suggestive of cerebellar dysfunction but at this stage there was no evidence of tremor, the head was continually ventroflexed, the pupils were dilated and the response to light was slow and incomplete.

When normal cats are held suspended by their hind limbs the head is dorsoflexed. In the critical stage, however, cats suspended in this manner kept their heads strongly ventroflexed, with the chin in apposition to the sternum. They were also unable to jump off the edge of a table, because the strong ventroflexion of the head caused them to somersault as soon as the head was over the edge of the table.

Convulsions were a transient feature of the first 24 hours of the critical stage. Their duration rarely exceeded 15 seconds and they occurred at short but irregular intervals of a few minutes apart. Convulsions could be easily provoked at this stage by handling the animal. If an animal was turned on its side during a convulsive seizure, the direction of the force was such as to cause complete flexion of the trunk so that the cat resembled a curled caterpillar. If maintained in sternal recumbency during a convulsion, the force pressed the animal firmly to the floor. From this stage there was rapid progression to limb spasticity, the animal appearing to walk on its toes, and to spinal hypersensitivity so that the stimulus of a sharp tap on the back might result in a sudden extensor thrust. Visual and auditory reflexes were retained but depressed. The optic and labyrynthine righting reflexes were absent. Body righting reflexes acting on the head were usually retained but an occasional animal, if placed upon its back, would remain in that position with the head ventroflexed and the limbs extended. Ophthalmoscopically the retinal veins were dilated and retinal hemorrhages were sometimes present.

Cats in this critical phase responded quickly to injected combinations of the B group vitamins although a degree of ataxia persisted in some of them. The pattern of response to treatment was essentially similar to that obtained in human Wernicke's disease (Cogan and Victor, 1954). If untreated there was rapid progression to the terminal irreversible phase of semi-coma, continual crying, opisthotonus and maintained extensor tone.

Pathology

The lesions, specific for the syndrome described, were confined to the central nervous system. Other tissues and organs showed the non-specific changes of malnutrition and dehydration. Ulceration of the pyloric mucosa occurred and was remarkable because of the rarity of ulceration in cats dying of other causes. The brain and spinal cord were examined histologically in all of the cases, and the eyes and peripheral nerves in four cases. Lillie's (1952) method for normal and degenerating myelin and Bodian's protargol method for nerve fibres did not reveal any alteration in the white matter of the spinal cord or in the peripheral nerve roots in acute cases.

In one cat the eyes had cysts of the iris and ora serrata, and atrophy of the retinas and optic nerves. The cyst formation is a common senile change in cats and dogs, and there is no basis on which to relate the atrophy to any specific cause since it occurred in only one cat, whose visual status prior to developing the thiamine deficiency syndrome was unknown. In a second cat there was gliosis of the optic disc and optic fibre layer of the retina and congestion of vessels in the retina and ciliary body. Severe passive congestion in the retina and ciliary body was the only change found in the two other sets of eyes that were sectioned. None of the sections happened to include the small retinal hemorrhages that were seen ophthalmoscopically.

The lesions present in the brain in acute cases were typically focal, bilaterally symmetrical haemorrhages in the periventricular grey matter (Figures 1, 2). Considerable variation occurred in the local extent of each lesion and in the number of nuclear masses involved. If the focal haemorrhages were of sufficient magnitude to be macroscopically visible they were found in the inferior colliculi, in which location the only consistent and most severe involvement occurred. Next to the inferior colliculi and in decreasing order of frequency lesions were found in the medial vestibular, lateral geniculate, habenular and oculomotor nuclei, in the accessory vestibular, cuneate and red nuclei, and, irregularly, in the mammillary bodies, the accessory cuneate nucleus, the superior colliculi and the commissures of the nucleus reuniens and ciliary nucleus. When, as was occasionally the case, the cerebral cortex was involved, haemorrhages were found in the medial and small pyramidal cell layers of the occipital cortex. In individual instances of the most severe development of lesions all of the above nuclei were affected, but with the exception of the consistent involvement of the inferior colliculi and the bilateral symmetry, there was no detectable pattern of involvement.

Microscopically, the lesions presented any one or combination of three features. Most frequently and typically there was varicose dilatation of the blood vessels with focal extravasations. In what were presumed to be less acute or more slowly developing lesions haemorrhages were present in some nuclear groups and oedema in others, or the two were found to coexist in the one nucleus (Figure 3). The third type of lesion was a reparative one and consisted of vascular hypertrophy, gliosis and "gitter" cell formation (Figure 4). Hemosiderin deposits were present in areas of old haemorrhage (Figure 6).

The vascular dilatation, which affected the veins especially, was not a localised phenomenon but part of a general venous dilatation and congestion within the brain and spinal cord, and was seen ophthalmoscopically and microscopically in the retina. The haemorrhages occurred predominantly from dilated veins in the central area of a nuclear group and tended to be confined within the limits of the nucleus. If the surrounding fibre tracts were involved it was invariably by extension of the extravasated blood through the

parenchyma from an intranuclear locus. The reparative phenomena of thickening of the perivascular reticulum and hypertrophy and hyperplasia of the endothelial nuclei were of later occurrence and gave the impression of neovascularisation. Whether or not new blood vessels form is uncertain but if Scharrer's (1939) finding that in the cerebellum each capillary is responsible for the parenchyma of 25 microns radius can be transposed to the nuclei under consideration here it is necessary to conclude that new blood vessels do not form and to support the view of Alexander (1940) that they merely become more visible by endothelial and adventitial hyperplasia.

The occurrence of interstitial oedema which involved, with the same bilateral symmetry, those nuclear groups in which haemorrhages may occur was also remarkable and suggested possible connotations for the pathogenesis of the lesions. The oedema was much more strictly confined to the grey matter than were the haemorrhages except in limited instances as, for example, in the superior colliculi where the grey and white strata were indefinitely delimited. The oedema, where blood vessels were visible, was typically perivascular and in cats in which the critical and terminal phases of the clinical syndrome were of the usual 3 to 4 days duration, degenerating myelin could not be demonstrated in these oedematous nuclei by Lillie's (1952) method for normal and degenerating myelin.

The reparative glial and mesenchymal responses were with one exception as are usual for the central nervous system. The exception was the presence of a prominent glial proliferation in nuclei which had apparently escaped injury as judged by failure to demonstrate any evidence of previous structural change, haemorrhage, oedema or demyelination (Figure 5). This gliosis was much more intense than the comparable lesion described by Street, Zimmerman, Cowgill, Hoff and Fox, (1941) occurring in a dog with experimental thiamine deficiency. The cellular mechanisms for the clearance of debris from the nervous system are notably sluggish and it is highly improbable that any tissue destruction or detritus, sufficient to provoke an extensive glial response, could be removed without trace. Further, the extent of the reaction was greatly disproportionate to any possible undetectable degree of myelin damage and blood pigments were still present in the adventitia of blood vessels of other nuclei. Occasional lightly pigmented histiocytes were present in the vascular adventitia in areas in which the glial reaction had occurred. Although the pigment was not extracted in paraffin sections, it did stain with oil-red-O in frozen sections and is probably the lipoprotein common to the vascular adventitia in normal cats.

Acute neuronal changes did not occur within the affected nuclei. In brains fixed by infusion through the carotid arteries under deep pentobarbital sodium anaesthesia and stained for Nissl granules there was perfect retention of the structural integrity of the neurons. Ultimately many of the neurons which are surrounded by

haemorrhage and oedema degenerated and were lost, but in recovered cases neurons outside the periphery of the lesion were normal in appearance. In the olivary nuclei, in which haemorrhage and oedema were not found, there was acute neuronal necrosis in the natural, but not in the experimental cases.

DISCUSSION

Inasmuch as signs of vestibular damage constituted part of the syndrome described here, it was necessary to distinguish the condition from streptomycin poisoning. Cats are very susceptible to the neurotoxic effects of this drug, so much so that they have been used as experimental animals in toxicity studies on the cochlear and vestibular systems (Berg, 1951; Hawkins and Lurie, 1953). The differential diagnosis was made on the basis of history, symptoms and lesions. Firstly, there was no history of streptomycin administration in any of the naturally occurring cases; secondly, the clinical signs were too diverse to be attributable to a purely vestibular lesion; and thirdly, while lesions involved the vestibular nuclei they were present also in other parts of the brain, whereas streptomycin damages the eighth nerve and its nuclei selectively.

The rôle which a deficiency of thiamine plays in the genesis of neurological disease has remained controversial. Perhaps the most careful evaluations of available evidence have been made by Cruikshank (1952) and by Follis (1948), who, with the nutritionists' awareness of the difficulties of pure deficiency experimentation and of the complexity of nutritional inter-relationships, have attributed to thiamine deficiency an important but not necessarily individually specific rôle in the genesis of neuropathies.

The clinical and pathological specificity of Wernicke's disease in man and its counterpart in other species is unequivocal but the validity of "thiamine deficient" diets in experimentally produced haemorrhagic encephalopathy remains subject to the limitations imposed by present knowledge of nutritional essentials and interrelations. Strong evidence for thiamine specificity is provided by its preventive and therapeutic efficiency in Chastek paralysis in foxes (Evans, Carlson and Green, 1942) and more recently by its therapeutic effect in Wernicke's disease of man (Cogan and Victor, 1954).

That cats fed diets deficient in thiamine or fed fish containing a thiaminase develop a typical clinical nervous disease which responds rapidly to injected crystalline thiamine is well established by the work of Everett (1944) and Smith and Proutt (1944). The naturally occurring disease described in this paper is analogous in its clinical manifestations to that described by these workers and the specificity of the associated lesions seems established by their development in cats fed a thiamine deficient diet and supplemented with the other vitamins of known neurotrophic effect.

The manner in which thiamine deficiency may occur naturally in cats is easily understood but most difficult to substantiate adequately for any given case. The distribution of a thiamine-splitting enzyme in fish has been extensively surveyed. Deutsch and Hasler (1943) examined a number of species from American waters and found enzymic activity in whitefish (*Coregonus clupeaformis*), Menomonee whitefish (*Prosopium cylindraceum*) freshwater smelt (*Osmerus mordax*), goldfish (*Carassius auratus*), creek chub, (*Semotilus atromaculatus*), fathead minnow (*Pimephales promelas*), sucker (*Catastomus commersonnii*), Channel catfish (*Ictalurus lacustris*), bullhead (*Ameirurus melas*), mud minnow (*Umbra limi*), white bass (*Lepibema chrysops*), sauger pike (*Stizostedion canadense*), and burbot (*Lota lota*).

Lieck and Ågren (1944) looked for thiaminase amongst 30 species of fish, both fresh water and salt water. They found the enzyme in ten species: bream (*Abramis brama*), "bjorkna" (*A. blicca*), "vimma" (*A. vimba*), carp (*Cyprinus carpio*), crucian (*C. carassius*), ide (*Leuciscus idus*), roach (*L. rutilus*), rudd (*L. erythrophtalmus*), tench (*Tinca vulgaris*) and garfish (*Belone acus*). Of these, the breams and garfish were caught in salt water. Similar findings were reported from Sweden by Ender and Helgebostad (1945) whose list included also sprat (*Clupea sprattus*) and herring (*C. harringus*), which are salt water fishes.

Tatarskaya, Fain and Larionova (1951) looked for thiaminase in various marine animals in the Black Sea and Tatarskaya, Kudryashov and Fain (1954) examined specimens in the White Sea. They found thiaminase in the following: the Black Sea shark (*Squalus acanthias*), and two species of sturgeon (*Acipenser stellatus* and *A. güldenstaedi*) in the Black Sea, and herring, skate (*Raja radiata*), smelt (*Osmerus operlanus*), flounder (*Pleuronectes flesus*) and the fish (*Anarhichas minor*) in the White Sea. Amongst fresh water fish in the Moscow area they found thiaminase in carp and in *Misgurnus fossilis* and *Alburnus alburnus*.

In a survey of American fresh water fish, Chaet and Bishop (1952) found thiaminase in *Hybognathus nuchalis regius, Leucosomus corporalis, Notropis bifrenatus, N. cornutus, N. hudsonius amaris, N. volucellus* and *Rhinichthys atratalus*. All of these are in the Family Cyprinidae, which contains the carp. The enzyme was found by Fujita (1954) in a variety of Japanese shellfish, of which clams had the most abundant amounts. It was present in oysters in scarcely detectable amounts. Fujita also reported the presence of the enzyme in crab and lobster. Amongst the Japanese fish containing thiaminase were Cruciate carp (*Carassius auratus*), carp (*Cyprius carpio*), grey mullet (*Mugil cephalus*), catfish (*Parasilurus asotus*), eel (*Anguilla japonica*) goby (*Acanthogobius flavimanus*) and several other species listed without common names.

Fujita stated that most of the Japanese fish that carried large amounts of thiaminase were fresh water fish and that the salt water fish he tested were generally found to be free from the enzyme. This is not the case for Scandinavian, Indian, Russian or American waters. Deolalkar and Sohonie (1954), who examined fish in India, found thiaminase in fresh water, brackish water and salt water fish. Suomalainen and Philgren (1955) in Finland found thiaminase in perch (*Perca fluviatilis*), pike (*Esox lucius*) and cod (*Cadus callarias*) as well as in other species that had already been reported.

The enzyme is present predominantly in the viscera of fish and Deutsch and Hasler (1943) found that in those species which contain the destructive

factor the thiamine of viscera and edible portions is destroyed under ordinary marketing conditions.

During the course of a year, as many as sixty species of fish taken on the Atlantic Gulf or Pacific coasts may be incorporated in the commercial cat food under consideration here, including Atlantic herring which are netted in very large numbers during certain seasons of the year. Not all batches of the cat food will lead to the development of encephalopathy and the variations in effectiveness are presumably related to the species of fish used in a particular batch. Supplements of cereal products are added before the fish meal is exposed to processing temperatures, and sufficient time may elapse for inactivation of the cereal thiamine when it is mixed with fish products containing the enzyme. As an indication of the possibilities in this respect, Melnick, Hochberg and Oser, (1945) reported that the addition of a raw clam suspension to a human diet resulted in marked destruction of the thiamine in vitro within 15 to 20 minutes.

An alternative manner for the destruction of thiamine is provided by the conditions of processing. Jansen (1954) states that thiamine is fairly resistant to high temperature and oxygen in a weakly acid medium but that in a weakly alkaline or neutral state the resistance to heat is not great. If processing of neutral foodstuffs is done at a temperature of 212°F or above, a substantial loss of thiamine takes place depending on the duration of heating. The pH range on ten cans of the commercial cat food subjected to 250°F for eight to ten minutes during processing, taken from ten different case lots, was 6.4 to 6.8.

The co-enzymic activity of phosphorylated thiamine in the metabolism of pyruvate tempts one to attribute the lesions of thiamine deficiency to local effects of accumulated pyruvate. It is not improbable that two more or less distinct functions of thiamine may ultimately be implicated in the genesis of the lesions. The observations of Binet and Minz (1934) and Minz (1938), confirmed and extended by von Muralt (1947), have established for thiamine a rôle in the transmission of the nerve impulse either directly cholineric or by inhibition of cholinesterase. From the fact that it is the pyrophosphate of thiamine that is active in carbohydrate metabolism and that this co-enzyme is replaced by thiamine itself or some other derivative of it in cholinergic activity, it is suggested that these two functions may be distinct, and an explanation is provided for the rapidity with which the early purely "biochemical lesion" may be reversed by injected thiamine and the more tardy return to normal of the blood pyruvate levels.

There is no available explanation for the pathogenesis of the structural lesion or the localisation to the periventricular grey matter. We can only agree with Campbell and Biggart (1939) who were the first to recognize and emphasize the probable significance of the aneurysmic dilatation of veins and capillaries in Wernicke's disease

and who wrote, "As regards the nature of the process, the vascular disturbance is, in comparison with the evidence of nerve cell damage, so marked and so constant that, in our opinion, it is the most important part of the process". In the thiamine deficient cats described the vascular disturbance is constant and as well as being present in those nuclei in which haemorrhage may occur, is also visible microscopically in the grey matter of the spinal cord and ophthalmoscopically in the retina. That Nissl substance and neuronal morphology is so well retained in cats killed in the acute stage in a manner precluding post-mortem artefacts is barely consistent with the concept of local pyruvate toxicity. Whether the oedema may be attributed to the endothelial changes alone or whether cerebral ground substance participates to some degree in its development cannot be decided as yet. That alterations of the ground substance do participate in the development of the lesions is strongly suggested by the occurrence of extensive astrogliosis without other lesions in the periventricular nuclei of treated and recovered animals, for as Hicks (1947) has shown the adequate stimulus to astrocyte proliferation is astrocyte injury.

The fact that acute neuronal changes occurred in the olivary nuclei of the natural but not of the experimental cases is deserving of comment. In his experiments with rats, Hicks (1955) found that the olivary nuclei were particularly susceptible to niacin deficiency. It may be that the olivary lesions in our cats were due to a niacin deficiency which accompanied the lack of thiamine, and that this deficiency was prevented in our experimental cases by the administration of niacin.

Although fish is popularly considered to be an excellent food for cats, the data reported here indicate that diets consisting largely of whole fish may actually be deleterious to the health of cats. Ours is not the first study pointing in this direction, since Coffin and Holzworth (1954) and Cordy (1954) attributed the occurrence of steatitis ("yellow fat") in cats to the feeding of commercial canned cat food consisting predominantly of whole fish. They considered the etiologic factors of steatitis to be a deficiency of vitamin E in the presence of large amounts of unsaturated fatty acids. In addition, Schnelle (1955) has found this type of diet to be a predisposing factor in dermatitis in cats. It is now the accepted practice to fortify canned evaporated milk with vitamin D, and it appears from the above that canned fish food should also be fortified at least with thiamine and vitamin E at some stage of processing subsequent to the cooking.

CONCLUSIONS

An account is given of a spontaneous disease of cats which affects some animals that are fed commercial cat food containing whole fish as their exclusive diet. The symptoms are comparable to those of Chastek paralysis in foxes and mink. The lesions are identical with

those of Chastek paralysis and very similar to those of Wernicke's disease of man. The condition can be produced experimentally and can be prevented by the concurrent administration of thiamine. It is thought to be a thiamine deficiency resulting from the inactivation of the vitamin by thiaminase which is present in the fish.

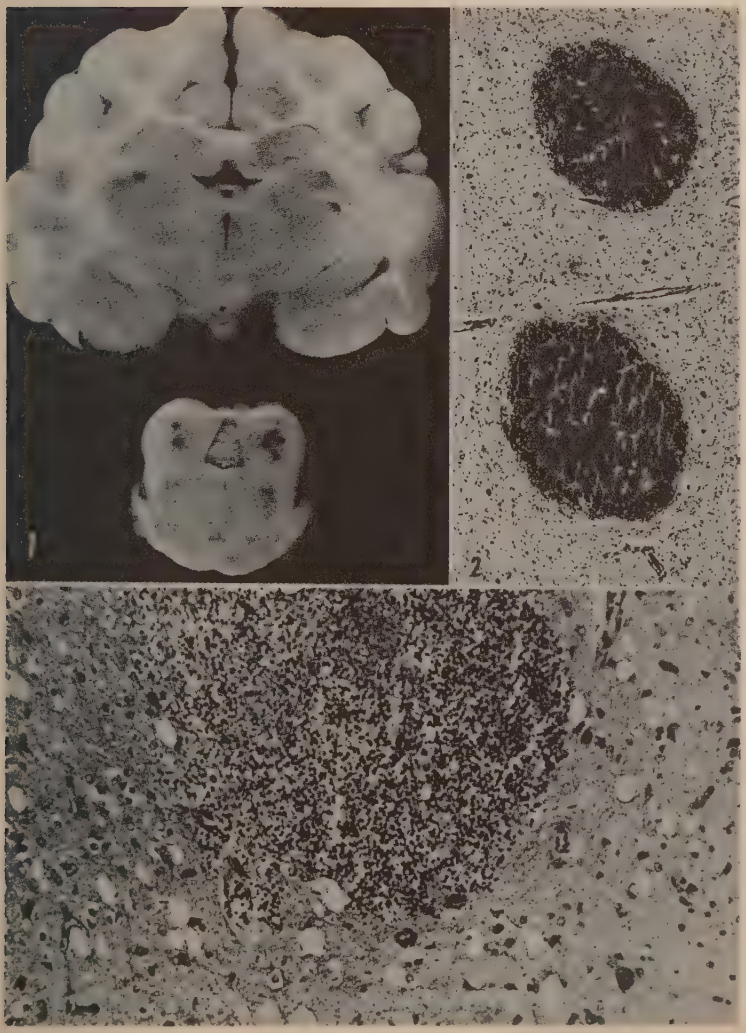
ACKNOWLEDGMENTS

We are indebted to Robert F. Smith, Brookhaven National Laboratory, for the photomicrographs.

A portion of this study was done at the Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

REFERENCES

Alexander, L. (1940). *Amer. J. Path.*, **16**, 61.
Berg, K. (1951). *Acta Oto-Laryngol.*, Suppl. 97, pp. 1-77.
Binet, L., and Minz, B. (1934). *Compt. r. Soc. Biol. Paris*, **117**, 1027.
Campbell, A. C. P., and Biggart, J. H. (1939). *J. Path. Bact.*, **40**, 245.
Carlström, B., and Jonsson, G. (1938). *Skand. vet. Tidskr.*, **28**, 144.
Chaet, A. B., and Bishop, D. W. (1952). *Physiol. Zool.*, **25**, 131.
Coffin, D. L., and Holzworth, J. (1954). *Cornell Vet.*, **44**, 63.
Cogan, C. G., and Victor, M. (1954). *A.M.A. Arch. Ophth.*, **51**, 204.
Cordy, D. R. (1954). *Cornell Vet.*, **44**, 310.
Cruickshank, E. K. (1952). *Vitamins and Hormones*, **10**, 1.
Deolalkar, S. T., and Sohonie, K. (1954). *Nature*, **173**, 489.
Deutsch, H. F., and Hasler, A. D. (1943). *Proc. Soc. exp. Biol. Med. N.Y.*, **53**, 63.
Ender, F., and Helgebostad, A. (1939). *Skand. vet. Tidskr.*, **29**, 1232; (1945) *Norsk vet. Tidskr.*, **57**, 32.
Evans, C. A., Carlson, W. E., and Green, R. G. (1942). *Amer. J. Path.*, **18**, 79.
Everett, G. M. (1944). *Amer. J. Physiol.*, **141**, 439.
Follis, R. H. (1948). *The Pathology of Nutritional Disease.* Charles C. Thomas; Springfield, Ill.
Fujita, A. (1954). *Advances in Enzymol.*, **15**, 389.
Green, R. G. (1936). *Minnesota Wildlife Dis. Invest.*, **2**, 106.
Green, R. G., Evans, C. A. and Carlson, E. W. (1937). *Ibid*, **3**, 173.
Hawkins, J. E., and Lurie, M. H. (1953). *Ann. Otol. Rhinol. Laryngol.*, **62**, 1128.
Hicks, S. (1947). *Arch, Path.*, **63**, 15; (1955). *Amer. J. Path.*, **31**, 189.
Jansen, C. P. (1954). In Sebrell, W. H., and Harris, R. S., *The Vitamins*, **3**, 455; Academic Press; New York.
Lieck, H., and Agren, G. (1944). *Acta. Physiol. Scand.*, **7**, 204.
Lillie, R. D. (1952). *Histopathologic Technique.* Blakiston; Philadelphia.
Long, J. B., and Shaw, J. N. (1943). *North Amer. Vet.*, **24**, 234.
Melnick, D., Hochberg, M., and Oser, B. L. (1945). *J. Nutr.*, **30**, 81.
Minz, B. (1938). *Compt. r. Soc. Biol. Paris.* **127**, 1251.
von Muralt, A. (1947). *Vitamins and Hormones*, **5**, 93.
Olafson, P. (1954). *N.Y. State Veterinary College.* Personal communication.
Pappenheimer, A. M., and Goettsch, M. (1931). *J. exp. Med.*, **53**, 11.
Prickett, C. O. (1934). *Amer. J. Physiol.*, **107**, 459.



Figs. 1, 2 and 3

To face page 226

Fig. 4

Fig. 5

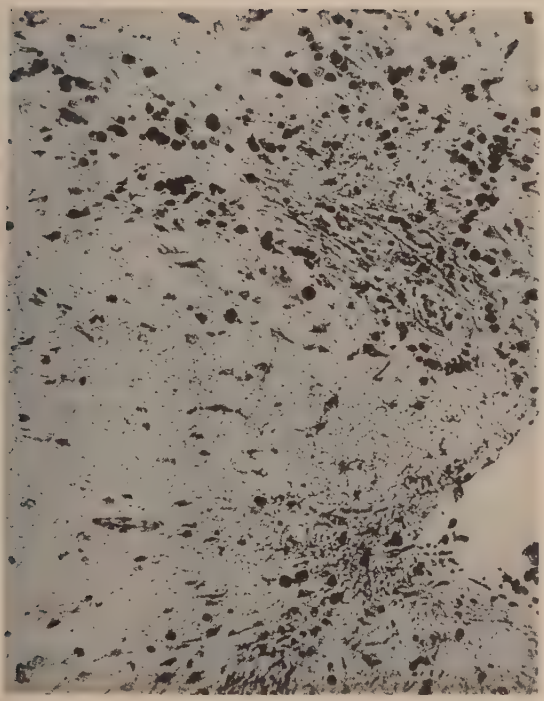


Fig. 6

Scharrer, E. (1939). *Anat. Rec.*, **75**, 319.
Schnelle, G. B. (1955). *Angell Memorial Animal Hospital, Boston, Mass.* Personal communication.
Sealock, R. R., Livermore, A. H., and Evans, C. A. (1943). *J. Amer. chem. Soc.*, **65**, 935.
Smith, D. C., and Proutt, L, M. (1944). *Proc. Soc. exp. Biol. Med., N.Y.*, **56**, 1.
Street, H. R., Zimmerman, H. M., Cowgill, G. R., Hoff, H. E., and Fox, J. C. (1941). *Yale J. Biol. Med.*, **13**, 293.
Suomalainen, P., and Pihlgren, A. M. (1955). *Acta Agralia Fennica*, **83**, 221.
Tatarskaya, R. I., Fain, F. S., and Larionova, L. P. (1951). *Biokhimiya*, **16**, 305.
Tatarskaya, R. I., Kudryashov, Y. B., and Fain, F. S. (1954). *Ibid.*, **19**, 229.
Woolley, D. W. (1941). *J. biol. Chem.*, **141**, 997.
Zimmerman, H. M. (1939). *Yale J. Biol. Med.*, **12**, 23.

[*Received for publication, December 12th, 1955*]

LEGENDS TO ILLUSTRATIONS

Fig. 1. Coronal sections through brain of natural case. Bilaterally symmetrical haemorrhages in the lateral geniculate nuclei (above) and in the inferior colliculi (below).

Fig. 2. Two haemorrhages in the inferior colliculus. H & E x 40.

Fig. 3. Area of haemorrhage and oedema in inferior colliculus. H & E x 200.

Fig. 4. Focal area of softening with oedema, vascular hypertrophy and "gitter cells" in medial vestibular nucleus. H & E x 250.

Fig. 5. Astrocytic proliferation in inferior colliculus. H & E x 55.

Fig. 6. Occipital cortex from cat treated with thiamine. Vascular adventitial hyperplasia and haemosiderin deposits in site of old haemorrhage. H & E x 120.

THE BIONOMICS OF THE FREE-LIVING LARVAE OF *DICTYOCAULUS VIVIPARUS*

By

J. H. Rose

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

The length of life of the free-living larvae of the cattle lungworm, *Dictyocaulus viviparus,* like that of all living organisms, is affected by a complexity of factors both biological and non-biological. To some considerable extent it must be governed by the microclimate, which is dependent upon the nature of the herbage as well as upon the general climatic conditions. In some earlier experiments (Michel and Rose, 1954), it was observed that in the autumn, larvae survived better on tall than on short herbage. This appeared to be due to differences in the microclimate resulting from the different lengths of the herbage. As it was reasonable to suppose that the form of the herbage plants might also have a bearing on the microclimate and on longevity, it was decided to compare the survival of larvae on grass and clover.

For the larvae to be of any significance in the epidemiology of parasitic bronchitis, they must be in a position on the herbage where they will be ingested by the grazing bovine. Some larvae may be so positioned when faeces are spread over the herbage by mechanical agencies, but it has never been satisfactorily demonstrated that they can migrate up the herbage from the faeces or soil surface. Daubney (1920) observed larvae migrating up the walls of glass culture vessels and Soliman (1953) demonstrated that they could move ½ cm. up a glass slide. Orlov (1946) maintains, however, that they do not migrate up grass blades. Taylor (1951) found that they did not exhibit any marked tendency to migrate upwards on moist surfaces under laboratory conditions, but thought that this might have resulted from the failure to reproduce conditions comparable to those occuring in the field. In view of these conflicting reports experiments were carried out to investigate this problem. Laboratory observations did not yield satisfactory results, but experiments using herbage grown in pots, which were kept out of doors, were more successful.

The present paper reports on these outdoor observations, and also on laboratory experiments designed to assist in their interpretation.

MATERIALS AND METHODS

Observations out of Doors

The longevity of larvae on white clover and Italian ryegrass. Experiments were carried out using infected faeces placed on rapidly growing herbage in boxes. In half of the boxes there were turves cut from a ley of white clover; in the other boxes the turves were of Italian ryegrass. At the beginning of each experiment the clover was approximately 6in. in height

and the foliage formed an almost complete cover. The ryegrass was a little higher and was dense. It was considered that the herbage was such as would provide favourable conditions for survival of the larvae. Some of the boxes were kept in cold frames to protect them from the rain and they stood in shallow trays filled with water, others were exposed in the open. Faeces were put on the boxes either as an undisturbed pat, or thinly spread over the herbage. The "undisturbed" faecal pat took the form of a central strip, six inches wide, right across the box. The "spread" faeces were distributed over the herbage by means of a spatula. Care was taken to ensure that as much as possible went on to the stems and undersides of the leaves of the clover, so that the larvae present would get the full benefit of the protection afforded by the foliage.

Each experiment was replicated on several boxes. At weekly intervals random samples were taken from one of the replicates. The box was divided into quarters, the herbage was cut from one quarter and the soil surface scraped to remove a layer approximately $\frac{1}{4}$in. thick. In addition, a 10 g. sample of faeces was taken from the undisturbed pats. The larvae were separated from the samples and enumerated in the way described in the paper already referred to.

One experiment was carried out during July to early September (summer) and another from October to March (winter).

Vertical migration. The methods used in both the experiments on vertical migration and the laboratory experiments were, except for two modifications the same as those employed in a study of the bionomics of the larvae of *D. filaria*. As a detailed account of these methods has been published already, Rose (1955), they will not be repeated in the present paper.

Laboratory Experiments.

Experiments were carried out to assess the effects of temperature and humidity on the longevity of larvae in faeces, the effects of temperatures below freezing point and desiccation on longevity, and the effects of temperature on the rate of development.

Wet faeces were examined at weekly intervals but dry faeces were examined at daily intervals for the first 14 days, then subsequently at weekly intervals. Separation of the larvae was effected by flotation, (Michel and Rose, 1954) and not by means of the Baermann apparatus, as was used for the separation of *D. filaria* larvae from sheep faeces.

RESULTS

The numbers of living larvae recovered from the faeces and herbage of the boxes kept out of doors, are represented in the Figs. 1 to 12. Very few larvae were recovered from the soil. The vertical distributions on grass blades as indicated by the numbers of larvae recovered from the different lengths of grass are recorded in Table I. The results of three experiments only are tabulated, as the pattern of vertical distribution was much the same in all of them. The results of the laboratory experiments are recorded in Tables 2 and 5.

When faeces and larvae were spread over the herbage there was a heavy initial mortality during the first week (Figs. 1 to 4). At the start of each experiment, approximately 3,000 living larvae were

Fig. 1

Fig. 2

Fig. 3

Fig. 4

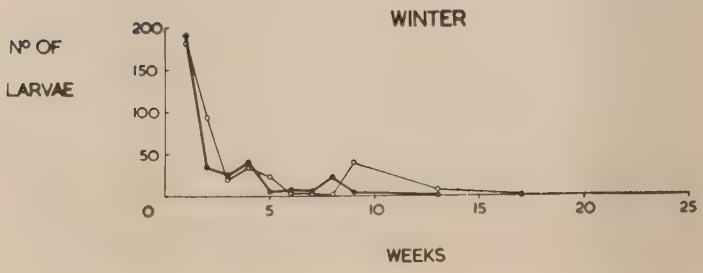


Fig. 5

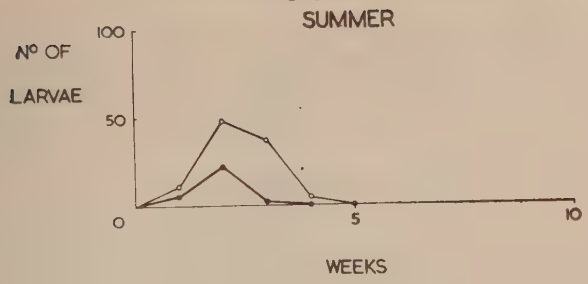


Fig. 6

Fig. 7

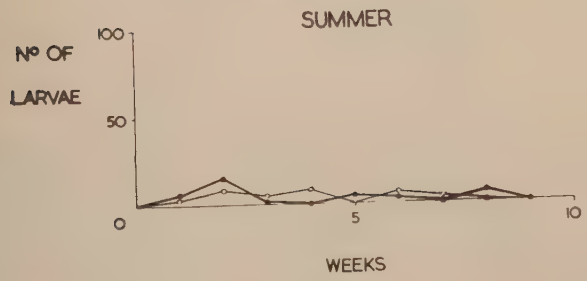


Fig. 8

spread over the herbage in one quarter of a box. By the end of the first week less than 200 living larvae were recovered from this area and many dead larvae were counted. The initial mortality was more marked in the summer than the autumn, but at no time was there any appreciable difference in the numbers of living larvae regained from the clover and the grass.

Few larvae passed from the faecal pats on to the herbage (Figs 5 to 8) except in the early winter on the exposed boxes (Fig. 6). At this time rainfall was heavy (Table 6) and the faecal pats on the exposed boxes were much wetter than those on the unexposed boxes. The unexposed pats remained moist where they were in contact with the soil, but the upper part dried out rapidly to form a thick hard crust which would act as a barrier to migrating larvae. It was undoubtedly as a result of the rainfall keeping the exposed faecal pats wet, thereby facilitating migration and possibly washing some larvae out of the faeces, that more reached the herbage in these than in the other boxes. There was, however, little difference in the numbers regained from the grass and the clover in boxes subjected to the same treatments.

TABLE 1

THE VERTICAL DISTRIBUTION OF *D. VIVIPARUS* LARVAE ON GRASS BLADES

Position on grass blades	*No. of larvae recovered*		
	Exp. 1	*Exp. 2*	*Exp. 3*
Top ½in.–1in.	0	0	0
Middle 1in.	0	0	0
Middle ½in.	15	21	9
Bottom ½in.	110	141	126

Larvae which reach the herbage were capable of migrating vertically, as can be seen from Table I. The extent of vertical migration was not very great, however, as none were found above the lowermost inch of the grass blades. This is not surprising as these larvae are never very active as are the larvae of many parasitic nematodes. When they are four to five weeks old, they are invariably found to be coiled up and subsequently exhibit very little activity.

The initial rate of mortality of larvae in the undisturbed pats (Figs. 9 to 12) was less than that amongst those distributed over the herbage (Figs. 1 to 4). During the summer, the initial mortality in the first week was more marked in the unexposed than the exposed faeces. This again appeared to be associated with differences in the faecal pats. During the first week there was sufficient rain to keep the exposed faeces wet, but the unexposed faeces dried out quickly except where in contact with the soil. The higher mortality in the exposed faeces was almost certainly due to this drying out. From the laboratory experiments it is clear that the larvae of *D. viviparus* were very susceptible to dryness (Table 3). No living larvae were

Fig. 9

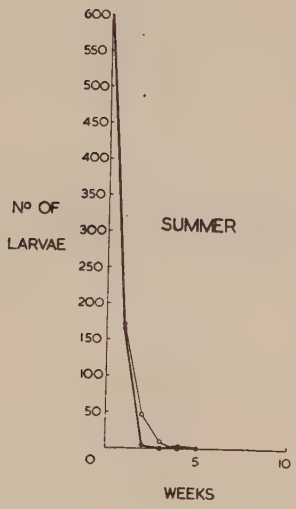

600
550
500
450
400
350
300
250
200
150
100
50
0
5
10
Nº OF
LARVAE
SUMMER
WEEKS

Fig. 10

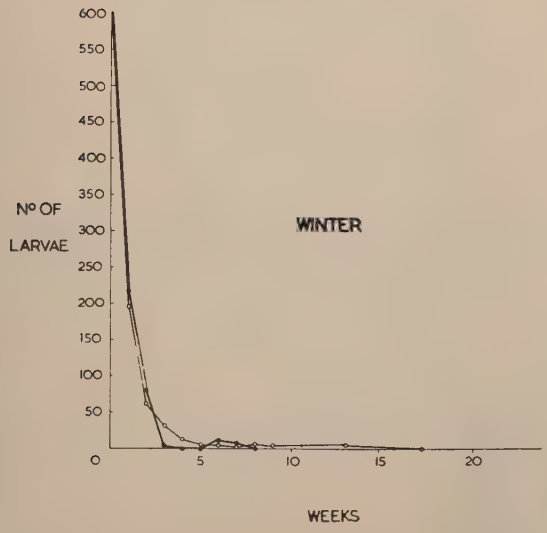

600
550
500
450
400
350
300
250
200
150
100
50
0
5
10
15
20
Nº OF
LARVAE
WINTER
WEEKS

Fig. 11

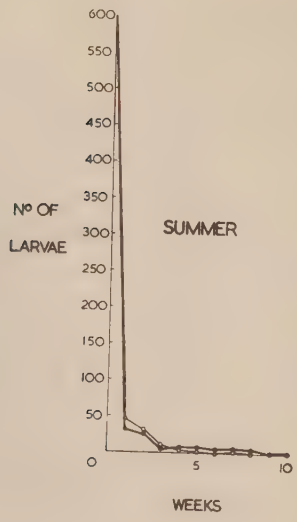
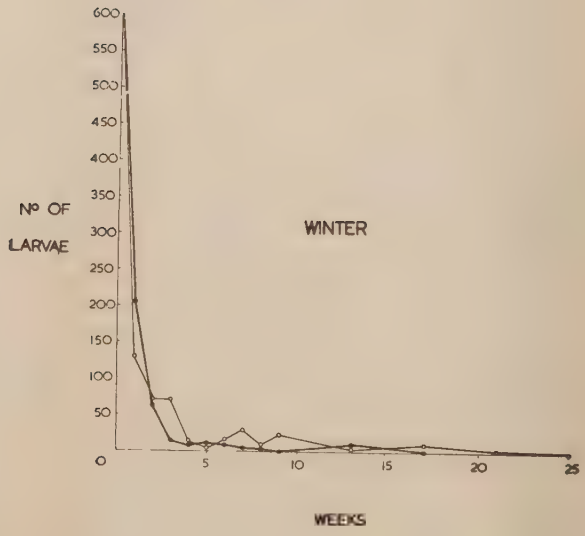

600
550
500
450
400
350
300
250
200
150
100
50
0
Nº OF
LARVAE
SUMMER
5
10
WEEKS

Fig. 12


600
550
500
450
400
350
300
250
200
150
100
50
0
Nº OF
LARVAE
WINTER
5
10
15
20
25
WEEKS

recovered after four weeks from dried faeces kept at 3 to 6°C and 14 to 22°C, while at 23 to 25°C longevity was further reduced. When dried out on slides, larvae succumbed rapidly; none survived 24 hours desiccation even at a relative humidity of 75 per cent. During the autumn, on boxes subjected to corresponding treatments, the initial mortality in faeces was less than in the summer, but there was no appreciable difference as between clover and grass.

TABLE 2

LONGEVITY OF *D. VIVIPARUS* LARVAE IN WET FAECES

Weeks	*Percentage of living larvae*		
	3-6°C	14-22°C	23-25°C
1	100	100	100
2	82	68	52
3	74	42	38
4	55	35	21
5	61	24	25
6	48	9	7
7	52	4	4
8	31	6	0
9	37	5	0
10	21	1	
11	15	0.4	
12	18	0.2	
13	11	0.2	
14	10	0.1	
15	5	0.1	
16	9	0.04	
17	4	0	
18	3	0	
19	5		
20	0.4		
21	0.2		
22	0.1		
23	0		
24	0		

Longevity was less during the summer than during the autumn, both in faeces and on herbage. Although this was mainly due to the drier conditions in the summer, some reduction in longevity would result from increased activity and metabolism which takes places as the temperature rises. Such a reduction did occur when larvae were kept in moist faeces at different temperatures in the laboratory (Table 2). At 23 to 25°C no living larvae were regained after 8 weeks, whereas at 3 to 6°C a few were still alive after 22 weeks. At the lower temperature range a few larvae out of several thousands survived for 12 months when kept in clean water. The potential life of these larvae is longer under favourable conditions than their actual life out of doors where the maximum period of survival observed was 5 months, from October 6th to March 2nd. In some other experiments, in which faeces and herbage remained undisturbed, a small number of larvae survived for 6 months during the winter and spring. It is

possible that a few larvae may have survived longer, as when only small numbers are left on the herbage recovery is difficult. The temperature was below freezing point on a number of occasions, but some larvae survived. This resistance to low temperatures was seen in the laboratory experiments, in which 2nd and 3rd stage larvae were alive after being frozen for several days (Table 4), but 1st stage larvae were quickly killed off.

TABLE 3

LONGEVITY OF *D. VIVIPARUS* LARVAE IN DRY FAECES

Days	*Percentage of living larvae*		
	3-6°C	14-22°C	23-25°C
0	100	100	100
1	73	38	36
2	65	21	21
3	62	22	20
4	50	23	14
5	54	15	8
6	31	12	2
7	27	11	
8	26	10	
9	21	8	
10	22	6	
11	21	3	
12	17	2	
13	16	2	
14	13	1	
21	3	0.8	
28	0	0	

TABLE 4

THE EFFECT OF FREEZING ON THE 1st, 2nd AND 3rd STAGE LARVAE OF *D. VIVIPARUS*

Period frozen Days	*Percentage alive 24 hours after removal from freezer*			*Control percentage alive*			*Temperature °C*	
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*Max.*	*Min.*
0	100	100	100		100			
1	0	11	17		,,		−2.1	−7.6
2	0	6	10		,,		−2.1	−7.2
3		2	6		,,		−2.4	−7.2
4		0.7	3		,,		−2.3	−7.2
5		0.3	1		,,		−2.3	−7.4
6		0	0.8		,,		−2.6	−7.8
7		0	0.5		,,		−2.7	−7.2
8			0		,,		−2.7	−7.4
9			0		,,		−2.3	−7.2
10			0		,,		−2.6	−7.4

In the outdoor observations, the larvae recovered at the end of the first week were at the third stage in both the summer and autumn.

As was to be expected, the rate of development of the larvae varied with the temperature (Table 5) and time of year. At low temperatures, development was surprisingly rapid. During January 1955, third stage larvae were recovered from faeces out of doors after only 4 weeks. In the laboratory at only 5°C, third stage larvae were observed after 26 days.

TABLE 5

EFFECT OF TEMPERATURE ON THE RATE OF DEVELOPMENT OF *D. VIVIPARUS LARVAE*

Temperature °C	*Minimum time in days to develop to*		
	2nd stage	*Granular 3rd stage*	*Clear 3rd stage*
5	16	26	31
10	7	10	12
15	3	4	5
20	2	3	4
25	2	3	4

TABLE 6

MEAN MONTHLY MINIMUM AND MAXIMUM TEMPERATURES AND TOTAL MONTHLY RAINFALL FROM JULY, 1954 TO APRIL, 1955

Month	*Temperature °F*		*Rainfall (inches)*
	Mean monthly minimum	*Mean monthly maximum*	
1954			
July	51.6	69.0	2.736
August	51.0	67.0	2.388
September	48.0	60.7	2.48
October	48.6	60.7	1.956
November	38.9	51.7	4.548
December	39.0	47.2	2.628
1955			
January	32.7	41.6	2.704
February	30.8	40.6	1.212
March	31.3	46.0	1.064
April	39.8	59.8	0.212

DISCUSSION

The impression gained from these observations is that larvae survive equally well on grass and clover if they are well grown. The broad leaves of the clover provide more shade than do the lanceolate leaves of the grass, but the clover does not grow so densely as grass. It would seem that the protection which clover foliage affords the larvae is no greater than that resulting from the denser growth of the grass. It appears, therefore, that on a well grown ley, larvae will survive equally well whether it is rich in clover or consists mainly of grasses.

The clover and grass did differ in one respect. When faeces were spread over the clover, the contaminated leaves were soon hidden by new shoots and within a few days the spread faeces were no longer visible. New grass shoots did not grow so rapidly, however, and the spread faeces were visible for a longer period. It is possible that the apparently clean clover would be ingested more readily than the obviously contaminated grass. At present, observations are in progress at Weybridge on the grazing behaviour of bovines, with particular reference to the extent to which they avoid contaminated herbage. It is hoped that this work will provide an answer to problems of this kind.

It is apparent that climatic factors have a profound influence on the longevity and rate of development of the larvae. Although it is the microclimate within the herbage which exerts a direct effect, this in turn is closely related to the general climatic conditions. From the laboratory experiments it is clear that dryness and high temperatures are unfavourable. These factors are largely responsible for the short longevity of larvae during the summer, but the effects of coprophagous insects and nematode-killing fungi, which are particularly active during warm weather, should not be ignored.

The longevity of larvae on pastures will be affected by both the nature of the herbage and the prevailing climatic conditions. Their potential longevity under favourable conditions has already been mentioned, a few out of several thousand larvae being still alive after 12 months. In their natural environment they are beset by many adverse factors and the actual longevity is unlikely to reach the potential longevity. In the present observations, which were made in 1954, a wet year, a few larvae survived for 6 months. In earlier observations (Michel and Rose, 1954) the maximum longevity observed was 13 weeks. A number of workers have endeavoured to assess the longevity of larvae indirectly by turning young parasite-free animals on to pastures which had been grazed by infected animals some months earlier. In Florida, Porter, Swanson and Drake (1941) turned yearlings on to a pasture in April 1941, which was last grazed by parasitised animals in the previous November. The temperature ranged from 24° to 85°F and there were 18.05 inches of rain during this period. On post mortem examination no *D. viviparus* were found, but stomach and intestinal worms were present. They concluded that the lungworm larvae had failed to survive on the pasture but the larvae of the other worms had been able to survive. As a result of further experiments, Porter (1942), concluded that larvae survived for one but not for six or seven weeks during warm dry weather on well drained pasture. Michel and Parfitt (1955), working at Weybridge, found that young animals failed to become infected when turned on to pastures which had been vacant for 5½ months, from December to May in both 1952 and 1953. In the West of Scotland under cool, wet conditions Jarrett, McIntyre, Urquhart and Bell (1955), using this method and also turning calves on to

small paddocks which had been heavily dunged, demonstrated that very small numbers of worms were picked up by calves put on after 6, 9 and 12 months. It is clear that observations made in one area under a particular set of conditions cannot be applied indiscriminately to other areas or other conditions. As the workers in Scotland have pointed out it is difficult to assess the epidemiological significance of the small numbers of larvae which survive for long periods. Although some of their calves remained on the plots for 2 or 3 months they did not appear to build up the infection at all. In some, no adult worms were found although there were indications that worms had been present. It does not seem possible that the small numbers of larvae which do succeed in overwintering can be directly responsible for the outbreaks of parasitic bronchitis encountered in the late summer and autumn, which are so characteristic a feature of this disease. The importance of "silent carriers" in carrying the infection over from one year to another has been pointed out by Wetzel (1948). Michel and Shand (1955) are in agreement with this view and the workers in Scotland have demonstrated that animals may act as carriers over the winter. Further field experiments are clearly necessary to assess the epidemiological significance of the small numbers of larvae which succeed in overwintering and also the rôle played by the carrier.

It has been shown in these observations that the larvae can migrate vertically up grass blades thereby enhancing their chances of being picked up by the grazing bovine. As in previous observations however, only a small percentage of the larvae moved from the faecal pats, even when there was much rainfall. As these larvae remained in close proximity to the faecal pat, it is unlikely that they would be ingested, as bovines tend to avoid grazing the herbage around a pat. It does seem, as has been previously suggested, that large numbers of larvae only reach a position on the herbage where they will be ingested, when the faeces themselves are thinly spread over it.

CONCLUSIONS

Experiments with infected faeces placed on rapidly growing herbage in boxes showed that there was no appreciable difference in the longevity of larvae on well grown grass and clover.

Whilst only a small percentage of larvae moved from the faecal pats on to the herbage, more did so from wet pats than from those which dried out.

Survival which was less in the summer than in the winter appeared to result from the susceptibility of the larvae to dryness. In the laboratory, longevity was less in dry than in wet faeces, and when dried out on slides no larvae were alive after 24 hours exposure to partial desiccation.

The maximum longevity on wet faeces increased as the temperature was lowered. A few larvae survived for 12 months in water at 3 to 5°C.

A small percentage of larvae overwintered: they survived temperatures below freezing point both out of doors and in the laboratory.

The rate of development was slowed down as the temperature was lowered. At 5°C, 3rd stage larvae were first observed after 26 days, but at 25°C. they were observed after only 3 days. Out of doors during January 3rd stage larvae were observed after 4 weeks.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. E. L. Taylor for helpful criticism and advice. My thanks are also due to Mr. J. F. Michel for supplying larvae and for his interest in the work.

REFERENCES

Daubney, R. (1920). *J. comp. Path.*, **38**, 225.
Jarrett, W. F. H., McIntyre, W. I. M., Urquhart, G. M., and Bell, E. J. (1955). *Vet. Rec.*, **67**, 820.
Michel, J. F., and Parfitt, J. W., (1955). *Vet. Rec.*, **67**, 229.
Michel, J. F., and Rose, J. H. (1954). *J. comp. Path.*, **64**, 195.
Michel, J. F., and Shand, A. (1955). *Vet. Rec.*, **67**, 249.
Orlov, I. V. (1946). *Veterinarya*, **23**, 4.
Porter, D. A. (1942). *Proc. helminth. Soc. Wash.*, **9**, 60.
Porter, D. A., Swanson, L. E., and Drake, T. J. (1941). *J. Parasit.*, **27** (Supplement) 22.
Rose, J. H. (1955). *J. comp. Path.*, **65**, 370.
Soliman, K. N. (1953). *Brit. vet. J.*, **109**, 364.
Taylor, E. L. (1951). *Vet. Rec.*, **63**, 859.
Wetzel, R. (1948). *Mh. VetMed.*, **3**, 141.

[*Received for publication, December 13th, 1955*]

LEGENDS TO FIGURES

Figs. 1—4. Larvae recovered from herbage over which faeces had been spread. Figs 1 and 2 "exposed to rain". Figs. 3 and 4 "protected from rain".

At 0 weeks approximately 3000 larvae were present on a "sample" of herbage but this point has not been plotted.

•———• Grass. o———o Clover.

Figs. 5—8. Larvae which had reached the herbage from the faecal pads. Figs. 5 and 6 "exposed to rain". Figs. 7 and 8 "protected from rain".

Figs. 9—12. Larvae recovered from 10 g. of faeces. Figs. 9 and 10 "exposed to rain". Figs. 11 and 12 "protected from rain".

STUDIES ON HOST RESISTANCE TO DICTYOCAULUS INFECTION

II. Re-infection Experiments with *D. filaria* in Sheep

By

J. F. Michel

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

That an animal which has recovered from an attack of parasitic bronchitis appears insusceptible to reinfection is a point of common observation but one which has been recorded in the literature on surprisingly few occasions. Thus, Wetzel (1948) states that calves become resistant to infection, but do so less rapidly than yearlings. This author however does not distinguish between the different manifestations of resistance and it is not clear whether he was referring to the termination of the initial infection or to resistance to reinfection. Gregoire (1951) also refers briefly to acquired resistance. Kauzal (1934) did not succeed in reinfecting lambs with *D. filaria*. Porter and Cauthen (1942), on the other hand, succeeded in reinfecting a 4½ months old calf with *D. viviparus* while failing to do so in yearling cattle. Porter (1941) reports that he succeeded in reinfecting two calves. Smythe (1937) maintains that no cattle of any breed or age acquire any degree of immunity to lungworm infection.

Since the manifestations of resistance are of considerable relevance to epidemiology, a study of these manifestations was begun in 1950. The purpose of the present paper is to describe the results of some early experiments with *D. filaria*.

MATERIALS AND METHODS

The lambs used in these experiments were of Oxford Down type, reared on the ewe in indoor pens with concrete floors. The floors of these pens were littered with sawdust and brushed out daily. Only dry food was used.

Larvae of *D. filaria* were obtained from the faeces of experimentally infected lambs by means of the Baermann apparatus. They were washed, resuspended in water and aerated. In rather less than a week at room temperature the larvae developed to the infective stage. Old larvae are less infective than new ones and since in these early experiments it was not always possible to use fresh material the actual numbers of larvae administered on different occasions cannot be regarded as strictly comparable.

Rectal faecal samples were collected from the sheep three times a week and examined for the presence of larvae by a method resembling that of Wetzel (1930). The stems of 4″ funnels, fitted with pieces of rubber tubing and secured with pinch clips, were filled with cold water and 5 g. samples of faeces put into the water above a small square of muslin. The funnels were allowed to stand overnight and in the morning 2 cc were withdrawn into a watchglass and the larvae counted. Recovery by this procedure

Fig 1

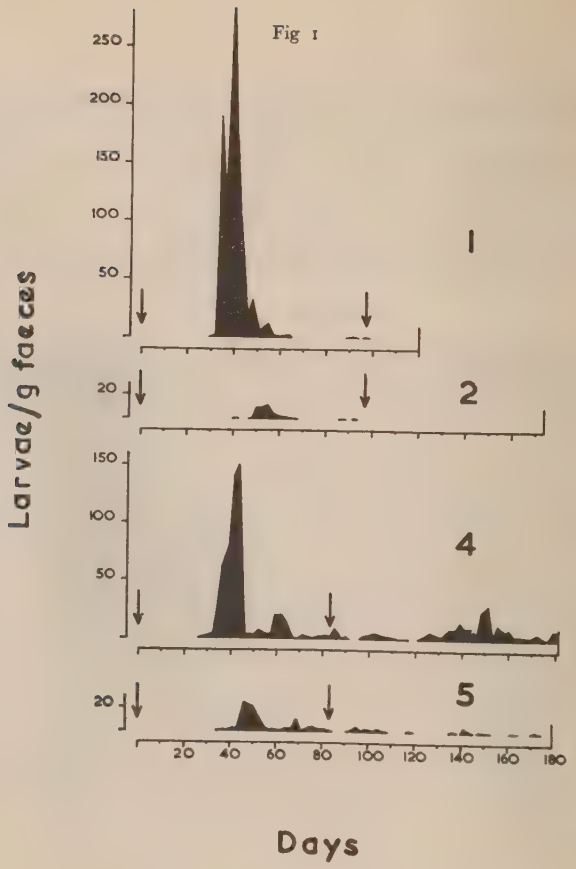
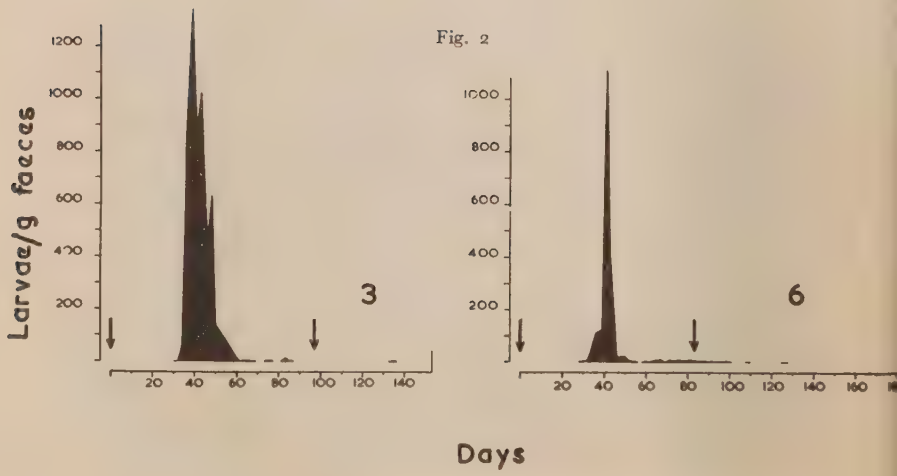


Fig. 2



Figs. 1 & 2. Faecal larval counts in Experiment I.
The administration of larvae to the lamb is indicated by vertical arrows.

was very satisfactory. Salt flotations, carried out on faeces after they had been subjected to Baermann extractions, showed that over 90 per cent are recovered by means of the funnels, some 80 per cent emerging from the faeces and into the water in the first five minutes.

The lungs of sheep were examined post-mortem for the presence of worms by a procedure already described elsewhere (Michel, 1954).

RESULTS

Experiment 1

Six six-month old lambs which had recovered from experimental infections of *D. filaria* were each given a large dose of larvae. The faecal larval counts of these animals are recorded in Figs. 1 and 2 and other details in Table 1. It would seem that the second infection failed to become patent in all but one animal (4) and even here the number of worms which could be attributed to the second infection was very small. None of these animals showed any symptoms as a

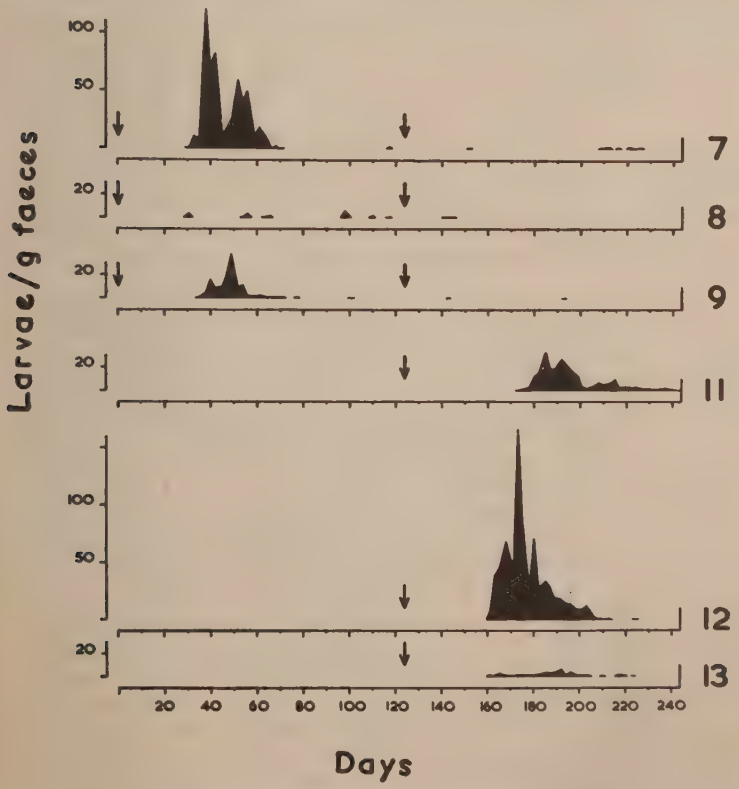


Fig. 3. Faecal larval counts of lamb in Experiment 2.
The administration of larvae is indicated by vertical arrows.

TABLE I*

Expt. No.	Animal No.	1st infection		2nd infection	Killed—days from last infection	Worms recovered post-mortem				
		Dose	Days from start	Dose		>3cm	>2cm	>1cm	>3mm	<3mm
I	1	6,000	97	80,000	24	—	—	—	—	—
	2	2,500	97	80,000	78	—	—	—	1	—
	3	30,000	97	80,000	57	—	—	—	4	—
	4	6,000	83	80,000	105	5	—	—	—	—
	5	1,000	83	80,000	98	—	—	10	—	—
	6	12,500	83	80,000	99	—	—	—	3	—
II	7	8,000	124	4,000	120	—	2	2	1	—
	8	4,000	124	4,000	120	—	—	2	—	—
	9	6,000	124	6,000	120	—	—	—	—	—
	10	—	124	6,000	Died 20	—	—	—	1,192	36
	11	—	124	4,000	120	2	—	1	—	—
	12	—	124	4,000	120	—	—	1	—	—
	13	—	124	6,000	120	2	1	4	—	—

*Details of Experiments 1 and 2.

result of the second infection. The response to re-infection was clearly very different from that to the first infection and it would appear that there had been some change in the susceptibility of the animals during the three months between the first and second infections. Whether this change was caused by the first infection or was due to the increased age of the animals cannot emerge from this experiment and accordingly a second experiment was undertaken.

Experiment 2

Three out of seven three-month old animals were infected and after 124 days all seven animals received moderate doses of larvae. Large doses were not used for fear of killing the previously uninfected animals. In spite of this precaution one of them died. The faecal larval counts of the six other animals are shown in Fig. 3 and other details of the experiment in Table 1.

The second infection did not become patent in the previously infected lambs but the response to this infection of the susceptible control lambs was much the same as that of the previously infected lambs to their first infection. The decreased susceptibility of the previously infected lambs was clearly due to their first infection and not to an increase in age.

The failure of the infection to become patent in resistant animals could be due to their failure to become established, their failure to persist sufficiently long to reach maturity, to the inhibition of their development or to the inhibition of ovulation. To throw a little light on this question a further experiment was undertaken.

Experiment 3, 4 and 5

Three lambs were infected and a further three were left uninfected. After 60 days all were given large doses of larvae. These were calculated on the basis of their bodyweights to allow for the fact that the uninfected lambs had grown more rapidly than the infected. One lamb from each group was killed after 14 days. One previously uninfected lamb died after 21 days and the remainder were killed on the 30th day. Details are given in Table 2 from which it will be seen that while fair numbers of worms were recovered from the animals which had had no previous experience of infection, only small numbers were present in the resistant lambs. It appears in the resistant animal that worms were absent not only after 30 days but also after 14 days, a finding which suggests that the worms failed to become established. Since this seemed a point of considerable importance two further experiments (4 and 5) were undertaken to provide adequate confirmation. Details of these experiments are also given in Table 2.

As these experiments could not settle the question whether the worms were prevented from reaching the lungs in the resistant animal a further experiment was planned to provide some evidence on this point.

TABLE 2*

Expt. No.	Animal No.	Age at start of expt.	1st infection	2nd infection		Killed—days from 2nd infection	Worms recovered post-mortem					Worms referable to 2nd infection	Remarks
			Dose	Days from start	Dose		>3cm	>2cm	>1cm	>3mm	<3mm		
III	14	11½ weeks	5000	60	69,000	14	—	—	21	23	2	2	2nd dose represents 1,500 larvae per lb. body weight
	15	9 ,,	5000	60	46,000	30	—	—	—	25	—	25	
	16	9 ,,	5000	60	40,000	30	50	—	—	90	—	90	1st infection still patent when killed
	17												
	18	9 ,,	—	60	77,000	14	—	—	—	—	1886	1886	
	19	9 ,,	—	60	80,000	21	—	—	—	3850	—	3850	Died
		9 ,,	—	60	61,000	30	444	72	226	28	—	770	
IV	20												
	21	10½ weeks	5000	117	96,000	15	—	—	—	—	89	89	2nd dose represents 2,000 larvae per lb. body weight
	22	14 ,,	5000	117	8,750	17	—	—	—	—	98	98	
	23	10 ,,	—	117	108,000	15	—	—	—	—	6934	6934	
		10 ,,	—	117	108,750	17	—	—	—	5660	89	5749	
	24	Between 18	5000	324	157,500	16	—	—	—	—	43	43	
	25	and 24 mos.	5000	189	151,500	20	—	—	—	—	74	74	
	26	at time of	—	—	246,000	15	—	—	—	—	9852	9852	
	27	2nd infection	—	—	262,000	18	—	—	—	—	5093	5093	
V	28	26 weeks	4000	112	40,000	14	—	—	—	—	319	319	
	29	24 ,,	4000	112	40,000	16	—	—	—	—	270	270	
	30	22 ,,	4000	112	40,000	18	—	—	—	9	24	33	
	31	29 ,,	—	112	40,000	15	—	—	—	—	4308	4308	
	32	22 ,,	—	112	40,000	18	—	—	—	—	2459	2459	
VI	33	24 weeks	4000	88	50,000	7	—	2	4	2	—	2	
	34	28 ,,	4000	88	50,000	9	—	—	—	4	21	21	
	35	27½ ,,	4000	88	50,000	14	—	—	9	—	88	88	
	36	27 ,,	4000	88	50,000	15	—	—	—	—	47	47	
	37	28 ,,	—	88	50,000	7	—	—	—	—	397	397	
	38	23 ,,	—	88	50,000	9	—	—	—	—	6110	6100	
	39	27½ ,,	—	88	50,000	14	—	—	—	—	4180	4180	

* Details of Experiments 3, 4, 5 and 6.

Experiment 6

Resistant lambs and their susceptible controls were given a large dose of larvae and one animal from each group was killed 7, 9 and 14 days later. The numbers of worms recovered from them are shown in Table 2. On the 7th day the larvae were apparently still arriving in the lungs of the susceptible lambs while by the 9th day they had all arrived. Meanwhile only very small numbers were arriving in the lungs of the resistant lambs. These results suggest that the worms fail to establish themselves in the lungs of resistant animals because they fail to reach them.

DISCUSSION

The evidence here presented would seem to demonstrate that lambs acquire a resistance to *D. filaria* which is due to previous experience of infection and not to increasing age. The principal way in which this resistance is manifest is by the failure of worms administered to the animal to become established in the lungs. This in turn appears to be due to the failure of the worms to reach the lungs.

From evidence presented in the first paper of this series (Michel, 1955) it will be seen that in the case of *D. viviparus* infections in the bovine, infections in animals that have had previous experience of infection fail to become patent and in animals killed at various intervals between 24 and 279 days after re-infection only very small numbers of worms were present.

The phenomenon whereby in the resistant animal worms are prevented from establishing themselves should be termed 'protection' the word used by Stoll (1929) to denote a similar phenomenon in infections of *Haemonchus contortus*. For the same reason the relatively abrupt termination of the initial infection seen in Figs. 1, 2 and 3 of the present paper should be termed 'self cure' as was the abrupt termination of his Haemonchus infections by Stoll.

One may think in terms of three manifestations of resistance to Dictyocaulus infection. 'Self-cure' abruptly terminates the infection by the elimination of adult worms. The second phenomenon 'protection', prevents worms from becoming established. The third, the inhibition of development, retards or entirely suppresses the growth of the worms. That these phenomena are closely paralleled in the case of other nematode infections is suggested by work on *T. retortaeformis* (Michel, 1952a, 1952b, 1953). While it is clear that the same manifestations of resistance occur in both *D. filaria* and *D. viviparus* it should not be assumed that their relative importance is the same in both cases. Thus it appears that in the calf the protection mechanism may be a little less effective than in sheep, since it is possible in some cases to produce symptoms in resistant cattle by the administration of larvae (Michel, 1954). Such infections do not become patent and the evidence suggests that though fair numbers

of worms may be initially established they survive in the lungs for only a short time.

CONCLUSIONS

When larvae of *D. filaria* are administered to sheep that have recovered from a previous infection, the worms fail to become established.

It is shown that this phenomenon is due to previous experience of infection and not to age.

Experimental evidence is presented which suggests that in the resistant animal the worms fail to reach the lungs.

REFERENCES

Gregoire, C. (1951). *Ann. med. Vet.*, **95**, 481.
Kauzal, G. (1934). *Aust. vet. J.*, **10**, 100.
Michel, J. F. (1952a). *Nature*, **169**, 933; (1952b). *Ibid.*, 881; (1953). *Ibid.*, **172**, 312; (1954). *Vet. Rec.* **66**, 381. (1955). *J. comp. Path.*, **65**, 149.
Porter, D. A. (1941). *J. Tennessee Acad. Sci.*, **16**, 357.
Porter, D. A., and Cauthen, G. E. (1942). *Amer. J. vet. Res.*, **3**, 395.
Smythe, R. H. (1937). *Vet. Rec.*, **49**, 1221.
Stoll, N. R. (1929). *Amer. J. Hyg.*, **10**, 384.
Wetzel, R. (1930). *Dtsch. tierärztl. Wschr.*, **38**, 49; (1948). *MhVet. Med.*, **3**, 141.

[*Received for publication, December 14th,* 1955]

THE AEROBIC INTESTINAL FLORA IN JOHNE'S DISEASE*

By

Ann Hirch†

Department of Veterinary Pathology, University of Liverpool

INTRODUCTION

One of the interesting but, as yet, largely unsolved problems in the aetiology of Johne's disease is the presence of lesions, often extensive, which are sometimes found post-mortem in animals which have shown no obvious clinical symptoms. This observation, taken in conjunction with the rather sudden or intermittent clinical breakdown which may occur in other animals, suggests the existence of some 'trigger' mechanism, responsible for the development of the acute disease in cattle in which *Mycobacterium johnei* had previously been responsible for a non-clinical form of the disease. Various explanations, advanced to account for the clinical breakdown, include calving, changes in diet and/or environment and a mineral imbalance. As the bacterial flora of man and most animals is known to be influenced by changes in diet, it seemed possible that some alterations in the food might sometimes be the precipitating cause of clinical breakdown, conceivably acting through a change in the balance of the intestinal flora. It was hoped that counts of the proportion of *Bacterium coli* in relation to other organisms in the intestinal contents of Johne-infected and non-infected ruminants, might yield some interesting results.

Although a large amount of work has been published on the bacterial flora of the intestinal tract of man and dogs, that of ruminants, apart from the rumen itself, has received less attention. Six published papers have been found and those of Küthe (1915), Carpenter and Woods (1924), Smith and Orcutt (1925) and Smoluinski (1951) deal only with the presence of *Bact. coli* at different levels of the small intestine in relation to scouring in calves; Smoluinski also examined material from adult cattle. Tunnicliff (1932) investigated the intestinal bacterial flora of lambs and Watts (1938) that of normal sheep.

The number of samples examined from clinically affected bovines was rather small, as it was necessary to culture or refrigerate the material from the animals soon after death, to avoid errors due to post-mortem multiplication of the bacteria and the upward movement of the contents of the large intestine. The total aerobic flora and the number of *Bact. coli* at two or three levels of the small intestine, have been determined on control animals, on cattle naturally and experimentally infected and on experimentally infected goats. Faeces samples have also been examined from the same groups of ruminants, but not necessarily from the same animals.

*Part of a thesis for the degree of M.Sc. University of Liverpool.

† Present address: School of Tropical Medicine, Liverpool.

EXPERIMENTAL METHODS

Faeces. The faecal samples examined from goats were obtained manually and cultured soon afterwards. If there was any delay between taking the samples and their examination they were kept in the refrigerator. Most of the bovine samples were collected immediately after voiding and refrigerated, or cultured within a few hours.

The method of treatment was the same in all cases. One gramme of faeces was weighed out in a screw-capped universal bottle. After the addition of 20 ml. of normal saline the bottle and its contents were shaken vertically ten times and then allowed to stand on the bench for 15 minutes to soften the faeces and facilitate disintegration. The bottles were then reshaken. If the goat faeces were very dry the pellets were broken up with a sterile scalpel before the saline was added. The bottles were spun very gently for one minute to bring down the coarse material and 10-fold dilutions were then made using 1 ml. of the supernatant. Five drops (0.1 ml.) of two or three appropriate dilutions were spread over the surface of both a blood agar and a MacConkey plate, and incubated at 37°C for about 18 hours. The total number of colonies was estimated on the blood agar and the number of *Bact. coli* on the MacConkey plates.

Contents of small intestine. The intestinal contents at the different levels were weighed out and treated in the same way as the faecal samples, but the dilutions plated were lower. The duodenum and lower ileum were the areas most frequently sampled but the jejunum was included where possible. The consistency of the contents tended to vary; the duodenal and usually the jejunal contents were liquid and contained a varying amount of mucus, but the material from the ileum varied from a liquid to an almost solid form. The intestinal contents were removed from the animal and placed in sterile bottles as soon as possible after death, generally within an hour. The material was then kept in the refrigerator until cultured.

As the dilution necessary to secure a countable number of colonies varied with the different samples, the bacterial counts, corrected to give the number of organisms per gramme of faeces or intestinal contents, have been converted into logarithms to facilitate analysis of the results. As counts of log 2.30 were the lowest that could be estimated by the method used, all samples where 0.1 ml. of the original 1 in 20 suspension was sterile, have been grouped together as $<$log 2.30.

RESULTS

Cattle

Duodenum. The duodenal contents of 30 animals have been cultured, of which ten were considered to be affected with Johne's disease. Eight of these animals were clinical cases of the disease and *Mycobact. johnei* had been cultured fairly consistently from the faeces of the remaining two animals. Six of the clinical cases were natural infections and the remaining four animals had been infected by the intravenous injection of *Mycobact. johnei* culture. The material from these last four animals was of particular interest as they were part of an experiment consisting of ten animals, all kept under identical conditions, which eliminated some of the variable factors, such as diet and age, known to influence the intestinal bacterial flora.

Six of the control group were part of this same experiment but the infection had apparently failed to develop as the cultures from the faeces for *Mycobact. johnei* were either consistently negative or had been negative for some months prior to their slaughter. No lesions of Johne's disease were found in the intestines post-mortem but *Mycobact. johnei* was grown from some of the organs. The mean total count of the intestinal material from these animals was lower than from that of the series as a whole. The remaining 14 control samples were obtained at the abattoir from supposedly normal animals sent for slaughter.

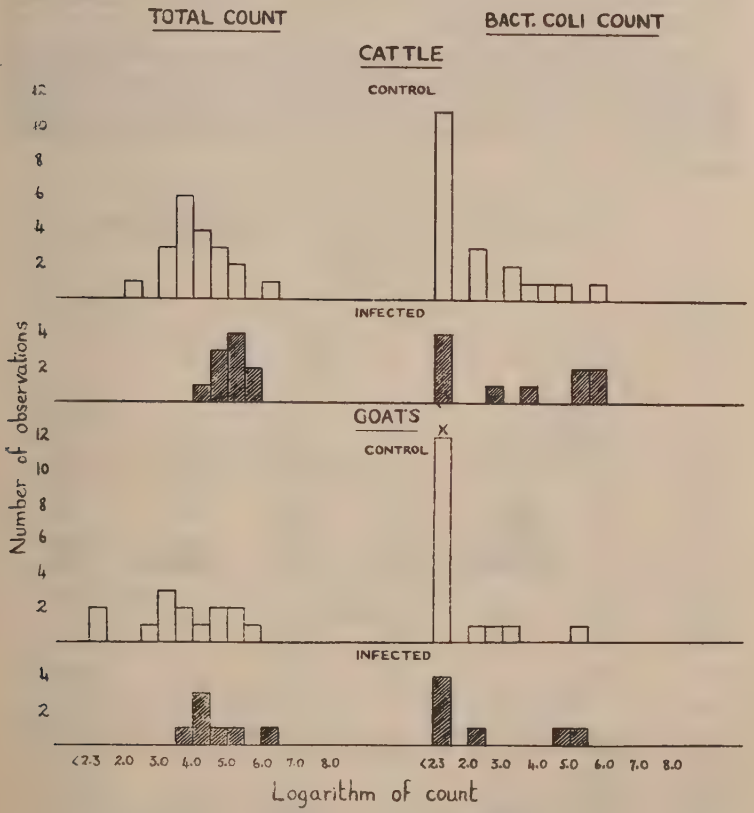


Fig. 1. Frequency distribution of the total and *Bact. coli* counts per gramme of duodenal contents.

Figs. 1 and 2. × The *Bact. coli* count was determined on two additional samples.

The grouped frequency distributions of the bacterial counts obtained have been drawn in the form of histograms (Fig. 1.). The ten samples from the infected animals have a mean total count of

log 5.06, while that of the controls is log 4.09. Although the number of observations was small this difference is statistically significant, being more than three times the standard error of the means. If the experimentally infected animals are considered separately the same difference occurs. The mean count for the four infected animals was log 5.18 and that of the six controls, log 3.58.

As will be seen from Fig. 1 no *Bact. coli*, at the lowest dilution plated, were found in a slightly larger proportion of samples from the control than the infected group, and there was a tendency for the counts to be lower. *Bact. coli* in pure, or almost pure, culture was found in four of the ten samples from infected animals, but only in four of the 20 control samples.

Ileum. Ileal contents were examined from the same ten infected animals and from two additional clinical cases. Control material was obtained from the six experimental animals but the 13 abattoir speciments were from animals whose duodenal contents were not examined.

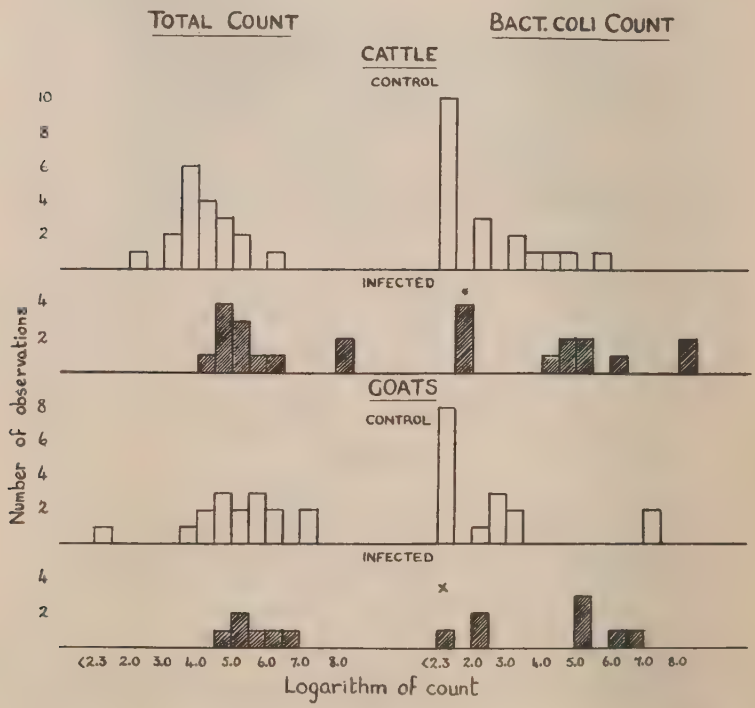


Fig. 2. Frequency distribution of the total and *Bact. coli* counts per gramme of ileal contents.

★ *Bact. coli* were absent from the lowest dilution plated, 10^{-1}.

In this case there was little difference between the mean counts of the two groups. The Johne-infected animals had a mean total count of log 5.60 and that of the controls was log 5.50. As the *Bact. coli* count of many of the samples can only be expressed as <log 2.30 no mean counts are available but, as will be seen in Fig. 2, a higher proportion of the samples from infected animals had *Bact. coli* counts greater than log 4.00. Eight of the twelve samples were pure, or almost pure, cultures of this organism.

One point of interest is the small difference between the total aerobic count in the duodenum and ileum of the infected animals. The findings of other workers would seem to indicate that in a healthy animal—and this is borne out to some extent by the counts obtained from control animals—a higher count would be expected in the ileum than in the duodenum. Results from 17 animals (10 infected and 7 controls) whose duodenal and ileal contents were determined are given in Table 1. The jejunal contents have also

TABLE 1

BACTERIAL COUNTS AT DIFFERENT LEVELS OF THE SMALL INTESTINE OF INFECTED AND UNINFECTED CATTLE

Reference number	*Log. count per gramme of contents*					
	Duodenum		*Jejunum*		*Lower ileum*	
	Total	Bact. coli	*Total*	Bact. coli	*Total*	Bact coli.
Clinical cases						
2848	5.23	5.06	—	—	6.11	6.06
2851	5.00	5.00	5.21	5.08	5.51	5.48
2825	4.89	2.60	4.56	<2.30	4.64	<2.30
2865	4.77	<2.30	5.08	3.81	4.85	3.60
2862	4.07	<2.30	4.20	<2.30	4.88	<2.30
M2	5.88	5.72	6.80	6.80	8.04	8.02
910*	5.30	<2.30	4.58	4.56	4.99	4.60
911*	4.53	<2.30	3.78	<2.30	5.30	<2.30
Faeces culture positive						
919*	5.64	5.53	6.06	6.06	4.30	4.30
918*	5.25	<2.30	4.72	4.30	5.03	4.88
Mean (10)	5.06		4.99		5.36	
(4)*	5.18		4.78		4.91	
Faeces culture negative						
915*	3.92	3.85	4.45	4.15	5.19	4.79
913*	3.84	2.30	4.15	<2.30	4.62	2.60
916*	3.30	2.30	—	—	4.62	4.53
912*	3.68	<2.30	—	3.58	5.56	5.02
914*	3.20	<2.30	4.03	<2.30	4.20	2.78
917*	3.53	<2.30	4.20	3.20	5.06	4.60
I	4.07	<2.30	—	—	5.87	<2.30
Mean (7)	3.65				5.01	
(6)*	3.58		4.21		4.87	

*Animals in infection experiment.

been included where they were available and in the control animals, but not in those clinically infected there is a gradual increase in the total count from the duodenum through the jejunum to the ileum.

Goats

Division of the samples of intestinal contents from goats into the infected and uninfected group was less clear cut than with the cattle. All the material available was from animals which had been given *Mycobact. johnei* culture under experimental conditions and, with one exception, the animals had been killed before becoming clinical cases of the disease so the degree of infection was difficult to assess. At the post-mortem examination films were made from scrapings of the mucosa of the small intestine at approximately three feet intervals. The number of typical clumps of acid-fast organisms were counted in a definite number of fields and they were then graded on an arbitrary scale from +++ to —. In the histograms (Figs. 1 and 2) the frequency distribution of the total and *Bact. coli* counts for those in groups +++ and ++ have been drawn separately from the rest. Their mean total count in the duodenum was slightly higher, log 4.52, as against log 4.14 for the others. (This is in fact an overestimate, as two of the samples which gave counts of <2.30 have not been included; if these samples were sterile the mean would drop to log 3.55). The ileal counts were higher in both groups, being log 5.69 and log 5.32 respectively. The counts in the duodenum and ileum of the one clinical case showed less difference, being log 6.09 and log 6.50; both were pure, or nearly pure cultures of *Bact. coli*. The *Bact. coli* counts tended to be higher in the ileal contents of the more heavily affected animals.

Faeces

The frequency distribution of the grouped faecal bacterial counts are shown in Fig. 3. The 14 infected bovine samples were from animals which were said to be cases of Johne's disease on the result of a faecal smear and in most cases there was confirmatory clinical evidence. Difficulty again occurred in grouping the material from goats, but only samples taken early in an experiment, before the animals had had time to develop the disease, have been included with those from uninfected animals. Faeces samples from these goats were not classified as being in the infected group unless diagnostic clumps of acid-fast organisms had been found in faecal smears, or the animal was visibly falling off in condition.

As can be seen in Fig. 3, there was little difference between the total bacterial counts of bovine faeces from infected and uninfected animals; the mean total counts were identical (log 6.43). There was rather more difference between the two groups of goats. The samples from uninfected animals had a more normal distribution and a mean count of log 6.56, while that of the infected group was log 7.04. The proportion of samples from infected goats which yielded a pure, or

almost pure, culture of *Bact. coli* was higher than from the infected cattle.

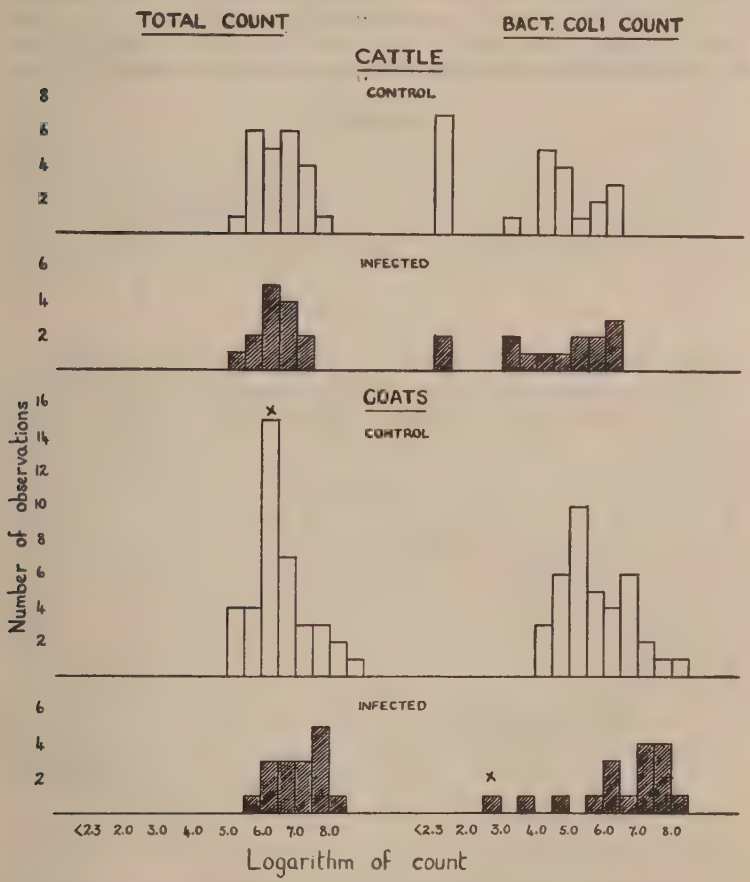


Fig. 3. Frequency distribution of the total and *Bact. coli* counts per gramme of faeces.
× There was one additional sample.

The Faecal Count in Relation to the Progress of Infection

At the beginning of this investigation it was found that several months after infection with *Mycobact. johnei,* the faeces of goats yielded pure cultures of *Bact. coli* to a fairly high dilution. Three uninfected goats receiving the same diet had a much more mixed flora containing a number of Gram-positive organisms. This observation seemed to indicate that infection with *Mycobact. johnei* might influence, or be influenced by, the other intestinal bacteria. The only method of

investigating this was by examining the faecal flora. In two subsequent experiments, in which goats had been drenched with cultures of *Mycobact. johnei*, faeces samples were cultured at approximately monthly intervals. Unfortunately, possibly due to a more nutritious diet, particularly in Experiment 2, these animals did not become infected as readily as those in the original experiment.

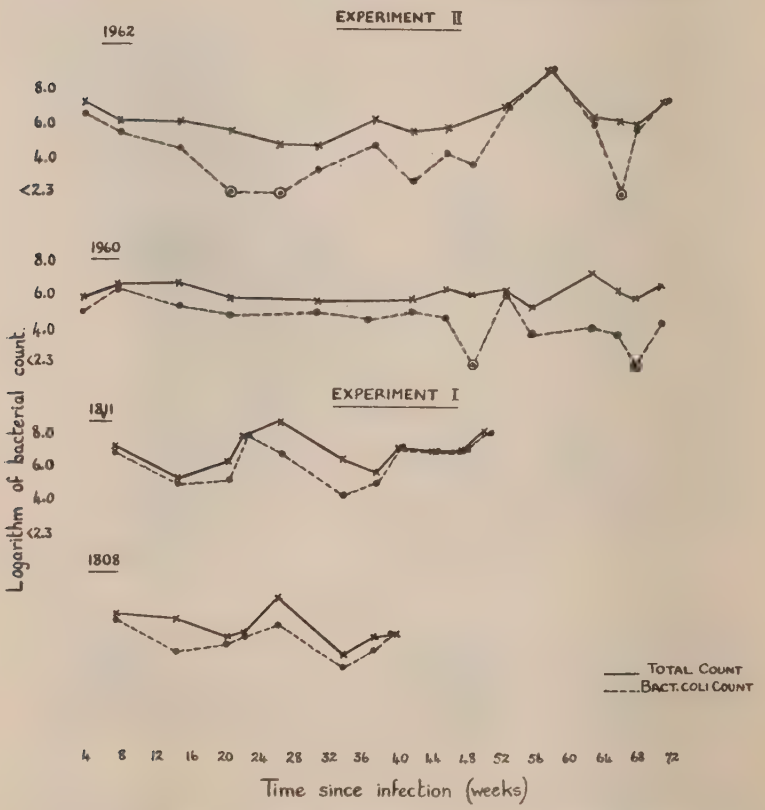


Fig. 4. Total and *Bact. coli* counts, per gramme of faeces, at intervals following the experimental infection of kids with *Mycobact. johnei*.

◎ This may be an underestimate as 10^{-1} was the lowest dilution plated.

In Fig. 4, the counts obtained from four animals are given graphically. Goat 1811 does show a fairly definite increase in both the number and proportion of *Bact. coli* in its faeces. 1808 was killed about 40 weeks after infection, just where the rise started in 1811, and there is an indication that the same thing might have occurred in 1808. The other two animals (1962 and 1960) are part of a dosage experiment. Four goats were kept for 70 weeks and only one of them,

(1962) which was losing weight and had rather soft faeces showed any symptoms of Johne's disease. At the same time it was the only one of the four to show any consistent increase in the proportion of its faecal *Bact. coli*. At post-mortem all four animals had lesions of Johne's disease in their small intestine and mesenteric lymph glands. There was little difference between the *Bact. coli* count in the ileum of 1962 and 1960; they were log 5.48 and 5.06 per gramme. *Bact. coli* was absent from the duodenal contents of both animals. The duodenal and ileal contents of 1811 gave a pure culture of *Bact. coli* with counts of log 5.48 and log 7.38 respectively. In addition, two of the animals from the original experiment, which gave high *Bact. coli* counts in their faeces on several occasions prior to their death, yielded pure, or almost pure, cultures of *Bact. coli* from their duodenal and ileal contents.

DISCUSSION

Although it was appreciated at the beginning of this investigation that the bacterial flora of the alimentary canal is subject to considerable variation, due to factors which could not be controlled, and that the procedure adopted, taking a weighed sample, was a possible source of error as the amount of solid matter in the samples would vary, it was felt that these factors would apply equally to all the samples and a comparison between the groups should be valid. In the case of the bovine intestinal contents the results obtained with the experimentally infected animals, which were the same age and kept under the same conditions, acted as a check on the series as a whole. It was, unfortunately, not possible to examine the species of bacteria isolated in any great detail, or to culture the material anaerobically.

The work reported here only covers a very small part of a complex problem. It is obvious that many more samples would have to be examined before a change in bacterial flora could be said to play a significant part in the clinical syndrome of Johne's disease.

The intestinal bacterial flora could affect the progress of infection with *Mycobact. johnei* in several ways. It could either assist the initial establishment of the acid-fast organisms and their subsequent multiplication in the intestinal wall, possibly by providing the necessary growth factor—or its precursor—as by-products of their metabolism, or, after infection is established, by changing the intestinal environment it might cause bacteria which have previously been existing as saprophytes to become pathogenic and produce the typical clinical symptoms of Johne's disease. Smythe (1950) suggested that the pathogenicity of Johne's bacillus is influenced by the bacteria with which it is associated.

The rise in the number and proportion of *Bact. coli* in the faeces and in the intestinal contents of some goats, which follows experimental infection with *Mycobact. johnei*, provides some confirmatory evidence for the presence of increased numbers of these organisms in

the alimentary tract of animals suffering from Johne's disease. The findings of Küthe (1915), Carpenter and Woods (1924), Smith and Orcutt (1925) and Smoluinski (1951), in calves, and Smoluinski in adult cattle, have shown that in sick animals, and this does not apply only to those suffering from diarrhoea, the upper reaches of the small intestine become invaded by organisms, chiefly coliforms, which are normally found in the lower ileum and caecum. Blaxter and Wood (1953) suggested that in some cases scouring in calves is secondary to a dysfunction high up in the alimentary canal; this results in defective absorption and undigested food residues are then available, which allow a massive bacterial multiplication. The metabolic products of these bacteria increase the osmotic pressure in the intestine, water is absorbed and finally the irritating watery mass is expelled. One of the causes of primary dysfunction cited is damage to the intestinal wall. It seems possible, therefore, that some of the symptoms of Johne's disease, particularly the scouring, which is often intermittent, could be due to a secondary infection of the small intestine, whose walls are damaged by the multiplication of the *Mycobact. johnei*. Elphick, in the discussion on a paper given by Dunkin (1929) suggested that the presence of lesions of Johne's disease allowed the multiplication of *Bact. coli* and that they were responsible for the very bubbly faeces characteristic of the disease. Elphick also instanced the bubbly faeces of a similar nature found in liver fluke infestations, an observation which might explain the findings of Smoluinski (1951) that, in adult cattle, the number of *Bact. coli* in the upper part of the small intestine was correlated with the degree of liver fluke infestation.

The only quantitative investigations of the bacterial flora at different levels of the intestine were those of Watts (1938) who used material obtained from healthy sheep at slaughter. The aerobic counts in the duodenum of these sheep were rather higher than would have been expected from the information available on other species of healthy animals and the mean count of the 15 samples (estimated as the number of organisms per ml. of contents) was higher than that of the normal goats and cattle examined here. The number and proportion of *Bact. coli* present in the duodenum of these sheep was also higher.

CONCLUSIONS

Estimates have been made of the total aerobic bacterial count, and of the *Bact. coli* count, of the contents at different levels of the small intestine of cattle and goats infected with Johne's disease and uninfected controls. Faeces samples were also examined from the same groups of animals.

The mean total count of the duodenal contents of ten Johne-infected bovines was log 5.06, while that of the 20 uninfected controls was log 4.09. The number of samples examined from infected animals

was small but *Bact. coli* was present, and in larger numbers, in a higher proportion of them than in those from uninfected animals.

The ileal contents of the 12 infected cattle had a mean total count of log 5.60 and that of the 19 controls was log 5.50. The *Bact. coli* counts again tended to be higher in the infected animals.

Similar findings were obtained with material from experimentally infected goats.

The mean total bacterial count of the faeces of infected and uninfected cattle was the same, *viz* log 6.43. Faeces from infected goats had a mean count of log 7.04 while that of the controls was log 6.56. The proportion of samples from goats which yielded a pure, or almost pure, culture of *Bact. coli* was higher than that from infected cattle.

ACKNOWLEDGMENTS

I should like to thank Professor R. E. Glover for his encouragement during the course of this work. My thanks are also due to Mr. T. M. Doyle, Mr. J. Deans Rankin and Mr. J. M. Olivant for making available to me material from infected and control cattle, and to Mr. R. M. Loosmore and Mr. W. E. Lawrence of the Veterinary Investigation Centre Liverpool, for collecting samples of bovine faeces.

This work was supported by a grant from the Agricultural Research Council.

REFERENCES

Blaxter, K. L., and Wood, W. A. (1953). *Vet. Rec.*, **65**, 889.
Carpenter, M., and Woods, Gladys. (1924). *Cornell Vet.*, **14**, 218.
Dunkin, G. W. (1929). *Vet. Rec.*, **9**, 915.
Küthe, H. (1915). *Zbl. Bakt.* (1 Abt. Orig.), **76**, 409.
Smith, T. and Orcutt, Marion L. (1925). *J. exp. Med.*, **41**, 89.
Smoluinski, H. (1951). *Inaugural Dissertation*, Munich.
Smythe, R. H. (1950). *Vet. Rec.*, **62**, 429.
Tunnicliff, E. A. (1932). *J. Amer. vet. med. Ass.*, **80**, 615.
Watts, P. S. (1938). *Vet. J.*, **94**, 60, 112.

[*Received for publication, December 28th, 1955*]

INFECTION OF HAMSTERS AND RABBITS WITH *MYCOBACTERIUM JOHNEI**

By

Ann Hirch†

Department of Veterinary Pathology, University of Liverpool

INTRODUCTION

Previous attempts to establish *Mycobacterium johnei* in small laboratory animals have been relatively unsuccessful. The most hopeful results have been obtained in mice (Boquet, 1925; Francis, 1943; Lominski, Cameron and Roberts, 1956) and in voles (Levi, 1950), but in this Department experiments in mice have been less encouraging. In the rabbit a light infection of the small intestine was obtained by Twort (1914) and Anderson (1922). Alikaeva (1940) found apparently typical lesions in the joints and pleura of rabbits 12 months after infection, but intestinal lesions were rare. Although hamsters are susceptible to infection with many Mycobacteria, Dunkin (1938) and Johnson and Pratt (1944) were unable to infect them with *Mycobact. johnei*. Francis (1943) tried to infect a small number of very young hamsters with 5 mg. of culture but the results were less encouraging than those obtained with mice. With the exception of Francis (1943) and Levi (1950), most of the earlier workers have used adult animals, and the infective material has been either intestinal scrapings from natural cases of the disease or cultures grown on the surface of solid medium.

With the object of testing further the susceptibility of laboratory animals experiments have been carried out in rabbits and hamsters. As susceptibility to infection in ruminants appears to decrease with age (Taylor, 1953) many of the trials, particularly with rabbits, were conducted on young animals. A further difference between these experiments and those of previous workers was the use of liquid cultures of *Mycobact. johnei* in which the organisms were younger.

METHODS

The cultures used for all these experiments were grown in a liquid medium (Glover, 1952), modified from that described by Dubos and Davis (1946). Among other changes the albumin V fraction of the latter medium was replaced by the supernatant obtained by treating bovine serum with Liquoid (sodium polyanethol- sulphonate). The Liquoid-treated serum was mixed with the appropriate amount of 10 per cent. Tween 80, sterilised by filtration, and then added aseptically to the basal medium to give a final concentration of 3 per cent. treated serum and 0.1 per cent. Tween 80.

As the media at present available are not suitable for doing viable counts, an estimate of the total number of organisms in the cultures was

*Part of a thesis for the degree of M.Sc. University of Liverpool.

† Present address: School of Tropical Medicine, Liverpool.

made using a photoelectric densiometer, similar to that described by Belyavin, Westwood, Please and Smith (1951). Several different strains of *Mycobact. johnei* were used, some of which had been recently isolated from goats, while others had been recovered from rabbits and hamsters infected in previous experiments. The age of the cultures varied but, particularly in the later experiments, an attempt was made to use a young actively growing culture not more than a week old.

Most of the rabbits were from four to six weeks old when infected. Young adult hamsters were orginally used but in some of the later experiments a whole litter was infected by the intraperitoneal route when only a few days old.

Feeding experiments. A measured amount of culture was fed to each animal from a pasteur pipette, the mouth being held open by a rigid bar held over the tongue. The hamsters tended to retain the culture in their cheek pouches, but most of it was eventually swallowed.

In the first rabbit experiments, the culture was diluted with milk but later it was used undiluted. The actual amount of culture fed in the different experiments varied slightly, but the majority of the animals received seven doses, each of 1.0 ml. of undiluted culture—or its equivalent in milk—on alternate days. Eleven of the 15 hamsters were given nine doses, each of 0.5 ml. of the undiluted culture, some daily and some on alternate days; the remaining four animals had only six doses.

Intraperitoneal injection. In the first three of these experiments (ten rabbits and six hamsters) the culture was mixed with an equal quantity of gastric mucin (Armour Laboratories) before being injected intraperitoneally into half of the animals; the remainder were given the culture diluted with an equal quantity of the liquid medium from which the Liquoid-serum—Tween had been omitted. The mucin was made up as a 10 per cent. solution in normal saline and sterilised by the addition of 1:10,000 merthiolate. When prepared in this way the final injection mixture was fairly thick and sticky; it could not be sterilised by the usual methods as after any form of heat treatment it became less viscous.

Two further experiments were carried out in hamsters. In one, using adult animals, the 'infectivity' of three cultures of *Mycobact. johnei* was compared; one strain had been recently isolated from a goat, one had been passaged through a hamster and the third through a rabbit. A similar comparison of strains with different histories was made in the last experiment in which three litters of young hamsters, two to three days old, were used. The first litter was given 0.1 ml. of culture intraperitoneally and the other two 0.2 ml. As the animals were very small the injections tended to be rather inaccurate, due to apparently unavoidable leakage from the abdominal cavity.

Intravenous injection. In two separate experiments a liquid culture of *Mycobact. johnei* was injected into the ear vein of ten young rabbits. The animals were given an initial dose of 0.5 ml. and then, nine or ten days later, a further dose of 1.0 ml. In each experiment half of the animals were injected with a strain fairly recently isolated from a goat and the other half with one which had been passaged through a rabbit and subcultured fairly frequently in the liquid medium.

Post-mortem examination. With minor exceptions, all the animals were examined in the same way at post-mortem. After noting details of any macroscopic lesions, or other abnormalities, the small intestine, from the

pylorus to the ileo-caecal valve, was opened and examined. From four to ten films, depending on the species of animal and the macroscopic changes seen, were made from scrapings of the mucous membrane. Films were stained by Ziehl-Neelsen and a large number of fields from each examined for typical clumps of *Mycobact. johnei*. Pieces of the liver, spleen, lung and kidney were removed for cultural and histological examination. In the later experiments a piece of the small intestine was included and the number of other organs sometimes reduced; the mesenteric lymph nodes of the rabbits were also cultured. In the early part of this work the egg medium advocated by Taylor (1950) was used but it was later replaced by the modified Dubos medium described by Smith (1953) as this had several practical advantages.

RESULTS

On account of the long period between the infection of a group of animals and assessment of the results there was some overlap of experiments. Certain points of interest have emerged, however, and these observations could serve as a basis for further work. The findings in rabbits were largely negative but they are described briefly as there is evidence of some slow multiplication of *Mycobact. johnei* in the organs of this host. The results obtained with hamsters, particularly after intraperitoneal infection, are more hopeful although, as yet, no clinical condition has been produced.

Feeding Experiments

Although the administration of *Mycobact. johnei* to small laboratory animals by the oral route had given consistently negative results in previous experiments it was decided to try it again, in parallel with other routes of infection, as it is the route of infection generally accepted as the natural one in cattle and it, therefore, seemed of interest to compare the distribution and survival of organisms in the organs of animals infected by this route with those given culture parenterally.

Hamsters. The 15 adult hamsters infected by this route died, or were killed, at intervals of from four and a half to forty weeks after their last infective dose. Nine of these animals, in two separate experiments, received a total dose of liquid culture containing the equivalent of approximately 1.5 mg. of moist organisms; the other four had only 0.6mg. Because of their very small size the mesenteric lymph nodes were not cultured. The organ which gave positive cultures most consistently was the liver; some colonies were grown from ten of the 14 examined. Positive cultures were also obtained from seven of the 13 lungs examined. There was some evidence that viable organisms tended to disappear gradually from the lungs. In one experiment positive cultures were obtained from the lungs of three animals killed 16 and 25 weeks after infection, but not from those of two killed after 40 weeks. In a previous experiment the lung cultures obtained from two animals which died four and a half and eight and a half weeks after infection were abundant but those dying

after 19 weeks yielded fewer colonies. Only one animal, killed after 40 weeks, yielded a positive culture from the kidney. A small number of typical clumps of *Mycobact. johnei* were seen in one or more of the intestinal films examined from three hamsters killed 40 weeks after infection. Intestinal films from other hamsters infected at the same time and killed earlier were all negative. Additional evidence of multiplication of the *Mycobact. johnei* following oral administration was provided by the one small experiment where four hamsters were given a total of 0.6 mg. of culture and killed after 6, 10, 20 and 40 weeks respectively. The cultures made from the organs of the first three animals were all negative, whereas a small number of colonies grew from the liver, kidney and intestine of the fourth killed after 40 weeks.

Rabbits. Repeated doses of liquid culture were fed to 22 young rabbits, mostly four to five weeks old, in five separate experiments, the total doses being equivalent to from 1.6 to 5.6 mg. Most of the animals were given culture of strain 3502, which had been isolated from a rabbit and repeatedly subcultured in the fluid medium. The remaining six animals were given a strain isolated from a hamster in another experiment. Although it was not realised at the time, the choice of these strains may have contributed to the disappointing results obtained.

The mesenteric lymph nodes and liver of several of these animals, killed up to 40 weeks after their last infecting dose, yielded some positive cultures but the number of colonies obtained was always small. A very small number of typical acid-fast rods were found in films made from the small intestine of four of these 22 animals: three of these, killed 25, 25½ and 36 weeks after infection, were animals given approximately 2.8 mg. of the hamster strain.

Intraperitoneal Injection

Hamsters. Two points of interest emerge from the results obtained by injecting small doses of a liquid culture into hamsters. On the one hand there is fairly definite evidence of the multiplication of the bacteria somewhere in the organs of this host and their subsequent establishment in the small intestine, although the process is slow: on the other hand there is apparently a variation in the 'infectivity' of different strains. Cultures isolated from goats, rabbits and hamsters have been compared, using, as far as possible, hamsters from the same litter. With the present methods the results obtained cannot really be compared on a quantitative basis, but in the later experiments the number of colonies appearing on the three to five slopes sown from the liver and intestine after treatment with oxalic acid were counted. Unfortunately, owing to losses, the animals were not killed at strictly comparable times following infection but the figures obtained in two experiments are given in Table 1; this shows both the increase in the number of colonies with increasing time since infection and the difference between the cultures. In the first

experiment the three cultures were grown in the liquid medium for the same length of time, *viz.* five days, but the density of the final culture varied, the animals given the goat strain receiving a lower weight of organisms than those given the passaged strains.

TABLE 1

INTRAPERITONEAL INJECTION OF YOUNG ADULT HAMSTERS WITH LIQUID CULTURE OF *MYCOBACT. JOHNEI*

Strain	*Dose (mg.)*	*Time after infection (weeks)*	*Culture Number of colonies per slope*		Mycobact. johnei *in intestinal films*
			Liver	*Intestine*	
1669 (from goat)	0.3	12½ K	0, 0, 0, 0, >100	18, 97, 29, 49, 0	None, 5/5.
		25 K	Very large	Very large	Moderate-large number of clumps, 4/6.
		40 K	„ „	Confluent growth	Moderate-large number of clumps, 4/6.
96 (from second hamster)	0.8	11½ K	99, 0, 0.	0,27.	Singles, 1/5.
		40 K	3,1,3.	91,35,77,57.	Singles, 1/6.
59B (from second rabbit)	0.5	12 K	0,43,12,7,84,	0,9,7,13,10.	Singles, 3/5.
		18 K	50,14,21.	Large	None, 8/8.
1812 (from goat)	0.3	3 D	1,0,0.	0,0,0.	None, 6/6.
		13 D	84,87,106.	32,34.	Very small number of clumps, 1/6.
		27 K	Very large	0, >100.	Small-moderate number of clumps, 6/7.
		35 K	„ „	Large	Small number of clumps, 1/6.
134 (from first hamster)	0.2	12 D	5,0,0.	N.D.	None, 6/6.
		19½ K	Large	Large	Singles, 1/6.
		35 D	0,0,0.	4,3,0.	Single, 1/6.

K — animal killed.
D — animal died.

A similar comparison of three strains with different histories was made in hamsters only two to three days old which were given approximately 0.1 to 0.2 ml. of culture—equivalent to about 0.04 to 0.08 mg.—by the intraperitoneal route. The four animals in each group (a group consisted of animals from a separate litter) were killed and examined at intervals from six to 35 weeks after infection. (Table 2). A small number of typical *Mycobact. johnei* were seen in films from scrapings of the mucosa of the small intestine of nearly all of these animals, but there was no real increase in the number until 26 to 35 weeks after infection. In this case strain 96, passaged twice

through hamsters, appeared to establish itself as well as that isolated from a goat. Strain 3502, originally isolated from a rabbit and subsequently subcultured many times in the liquid synthetic medium, showed no evidence of multiplication.

TABLE 2

INTRAPERITONEAL INJECTION OF VERY YOUNG HAMSTERS WITH LIQUID CULTURES OF *MYCOBACT. JOHNEI*

Strain	*Dose (mg.)*	*Time after last dose (weeks)*	*Culture Number of colonies per slope*		Mycobact johnei *in intestinal films*
			Liver	*Intestine*	
1809 (from goat)	0.08	6	0,0.	3,0,1.	Very small number of clumps, 2/6.
		15	10,0,0.	0,0,0.	One good clump out of 6.
		26	1,12,0.	75,114,>100.	Few singles, 2/5.
		35	19,2,35,20.	5,0,25,26.	Moderate number of clumps, 2/6.
96 (from second hamster)	0.04	7	0,0,2.	0,0,2.	Very small number, 1/6.
		16	0,0,0.	0,0,0.	None, 6/6.
		26	50,12,0.	Large	Moderate number of clumps, 2/6.
		35	2,0,0.	,,	Small number of clumps, 2/6.
3502 (from rabbit)	0.08	6	0,4,0	0,0,0.	Few singles, 1/6.
		15	0,0,0.	0,0.	Very small number of clumps, 3/6.
		24½	0,0,0.	10,0,0.	Small number of clumps, 1/6.
		35	0,0,0.	1,0,0,0.	None, 6/6.

In view of this result it seemed desirable to determine whether strain 3502, which appeared to be the least infective for hamsters, was still virulent for ruminants. A kid was given liquid culture orally, the total dose being equivalent to about 50 mg. of organisms; it failed to develop clinical symptoms and was killed after 11 months. At post-mortem examination, however, the mesenteric lymph nodes were enlarged and partly calcified and a moderate number of typical clumps of acid-fast bacilli were found throughout the small intestine. After repeated subcultures in the liquid medium this strain grew on the modified Dubos solid medium in the absence of an extract of *Mycobact. phlei*, but it did not grow on Lowenstein-Jensen or Dorset egg containing glycerol.

Rabbits. Ten rabbits, four to six weeks old, were injected intraperitoneally with approximately 0.2 mg. of organisms in 1 ml. of liquid medium. The first four were given the rabbit strain, 3502, and the remainder a strain isolated from a goat. The post-mortem findings were again influenced by the strain used. When the goat strain was injected positive cultures were obtained from the liver, mesenteric

lymph nodes and spleen of most of the animals. The number of colonies was always small and remained relatively constant after infection: on the other hand, a small number of typical clumps of acid-fast organisms were found in films made from the intestines of two animals killed after 30 and 42 weeks respectively, but not from those killed earlier. When the rabbit strain was used two rabbits killed 35 weeks after injection were negative.

Although the number of animals examined was small, injection of the bacteria suspended in a 10 per cent. solution of mucin did not seem to influence the results in either hamsters or rabbits.

Intravenous Injection of Rabbits

Two small experiments were undertaken to investigate the effect of the intravenous injection of liquid cultures on young rabbits and to compare the 'infectivity' of a goat and rabbit strain respectively when given by this route.

Ten animals, eight to ten weeks old, were given about 0.5 mg. of bacteria into the ear vein in two doses: each culture was given to half the animals in both experiments. They were killed at intervals from one to 33 weeks. A very small number of typical acid-fast clumps was found in the small intestine of one of four infected with the goat strain and killed about 32 weeks after infection. On the whole the difference between the strains was not so obvious in these groups. Positive cultures were obtained from the mesenteric lymph nodes and liver of all the animals but there was a tendency for the number of colonies appearing on the slopes to decrease as the period between infection and death increased.

DISCUSSION

These experiments, although confirming the relative avirulence of *Mycobact. johnei* for small laboratory animals, have shown that freshly isolated strains are able to multiply in the tissues of hamsters and, more slowly, in rabbits ultimately becoming established in the small intestine. Except for Levi, (1950) who used voles, the weight of organisms injected has been very much less than that used by previous workers.

Although typical acid-fast organisms have been demonstrated in the small intestine, liver and mesenteric lymph nodes, sometimes in large numbers, the animals appeared quite healthy and, apart from a slight thickening of the small intestine and enlargement of the mesenteric nodes, no obvious changes were seen at the post-mortem examination. The presence of a large number of organisms and extensive histological change without corresponding macroscopic changes was also found by Francis in mice and Levi in voles.

The sections made from the tissues of these animals have not been subjected to any very detailed histological examination, but the lesions found have been characteristic and are very similar in hamsters, rabbits and mice. They correspond to those found in many

cases of the experimental disease in goats in which, apart from the mesenteric lymph nodes and intestine where macroscopic changes do occur, extensive microscopic lesions can be found in the apparently normal liver, lung and kidney tissue (Harding, 1956).

On a weight basis the rabbits used in these tests had a relatively smaller dose of culture than the hamsters: moreover, many of them were given a strain re-isolated from a rabbit which seemed to be less able to multiply in the tissues of rabbits and hamsters than strains recently isolated from goats. Even when these factors were taken into consideration, the results obtained with young adult hamsters seemed to be more promising, as establishment of the bacteria in the small intestine was more rapid. It should be emphasised that rabbits were generally infected soon after weaning at about four weeks of age, and very young rabbits were not tested. Francis (1943), however, injected four ten-day old rabbits with 29.9 g. of culture and reported that smears from the organs and intestinal tract of three of them, killed after 27 weeks, were negative.

The apparent variation between strains in their ability to establish themselves, particularly in the small intestine of rabbits and hamsters, is of interest. The original chance observation that strains recently isolated from goats were recovered more readily from rabbits and hamsters than those passaged through these laboratory animals was confirmed in subsequent experiments. On the other hand, in the infection of very young hamsters, the strain passaged twice through hamsters appeared to be as 'infective' as that isolated from a goat. In all these experiments there seemed to be a 'lag' period, usually at least 25 weeks, before the organisms could be detected in the small intestine in stained films. In some later experiments, not yet completed, small numbers of typical organisms have, however, been found in intestinal films of young hamsters nine and ten weeks after the intraperitoneal injection of two strains of *Mycobact. johnei,* both passaged twice through hamsters. Clearly, more experiments are desirable using strains whose complete history is known, with due regard to standard conditions of dosage, age of culture, and number of subcultures on solid and in liquid medium respectively.

One characteristic property of *Mycobact. johnei* is its ability to exist in the intestinal tract of ruminants or laboratory animals, often for long periods, without apparently affecting the health of the animal. In ruminants, under certain conditions which are not yet clearly understood, the organisms become 'activated' in some way and clinical breakdown results, but no such occurrence has yet been seen in laboratory animals. On the other hand, none of these hamsters (the animal which seemed to be the most sensitive) was kept for more than 40 weeks, and it is possible that if they had been allowed to live, a pathological condition might have been produced. It is clear, however, that in the present state of our knowledge, the incubation period in these animals is too long for practical purposes. It might be profitable, nevertheless, to subject infected hamsters to

some of the conditions which have been said to produce clinical breakdown in cattle, such as parturition, a Ca – P imbalance, and changes in diet. Some increase in the virulence of *Mycobact. johnei* for hamsters might be induced by changing their metabolic state, using methods similar to those of Dubos (1955) who increased the infectivity of *Mycobact. tuberculosis* for mice in this way. Experiments are now being conducted in which hamsters are receiving a two per cent. sodium citrate solution instead of drinking water.

CONCLUSIONS

Attempts were made to infect hamsters and rabbits with small doses of a liquid culture of *Mycobact. johnei* generally the equivalent of 0.5 mg. or less. The oral, intraperitoneal and intravenous routes have been employed.

Hamsters, particularly those injected when only a few days old, appeared to be more susceptible than rabbits four to six weeks old. A moderate number of typical acid-fast rods were detected in films made from the small intestine of hamsters from about 25 weeks after infection. No clinical disease was produced in animals kept up to 40 weeks after infection.

There was evidence of a variation in the 'infectivity' of different strains for these laboratory animals; strains recently isolated from goats appeared to be able to establish themselves more readily than those previously passaged through rabbits and hamsters.

ACKNOWLEDGMENTS

I should like to thank Professor R. E. Glover for his advice during the course of this work. The Liquoid was kindly supplied by Roche Products Ltd. This work was supported by a grant from the Agricultural Research Council.

REFERENCES

Alikaeva, A. P. (1940). *Veterinariya Moscow*, No. 2. 26. Abst. *Vet. Bull.*, **14**, 149.
Anderson, C. W. (1922). *Arch. wiss. prakt. Tierheilk.*, **47**, 77.
Belyavin, G., Westwood, J. C. N., Please, N. W. and Smith, Wilson (1951). *J. gen. Microbiol.*, **5**, 546.
Boquet, A. (1925). *C.R. Soc. Biol., Paris.*, **93**, 219.
Dubos, R. J., and Davis, B. D. (1946). *J. exp. Med.*, **83**, 409.
Dubos, R. J. (1955). *Ibid.*, **101**, 59.
Dunkin, G. W. (1938). *Report 1st. Imperial Veterinary Conference*, Weybridge.
Francis, J. (1943). *J. comp. Path.*, **53**, 140.
Glover, R. E. (1952). *Proc. R. Soc. Med.*, **45**, 475.
Harding, H. P. (1956). *In press.*
Johnson, E. P., and Pratt, A. D. (1944). *Amer. J. vet. Res.*, **5**, 117.
Levi, M. L. (1950). *J. comp. Path.*, **60**, 10.
Smith, H. Williams. (1953). *J. Path. Bact.*, **66**, 375.
Taylor, A. W. (1950). *Ibid.*, **62**, 647, (1953). *J. comp. Path.*, **63**, 355.

Twort, C. C., (1914). *Veterinary News,* **11**, 79.

A paper by Lominski, I., Cameron, J., and Roberts, G. B. S. (1956). *J. Path. Bact.,* **71**, 211, came to hand after this article had been submitted for publication.

[*Received for publication, December 28th, 1955*]

A STUDY OF *SALM. PULLORUM* AND *SALM. GALLINARUM* STRAINS ISOLATED FROM FIELD OUTBREAKS OF DISEASE

By

J. D. Blaxland, W. J. Sojka and A. M. Smither

Veterinary Laboratory, Ministry of Agriculture, Weybridge, Surrey.

INTRODUCTION

Since the discovery of the causes of fowl typhoid and pullorum disease by Klein (1889) and Rettger (1900) respectively, most veterinarians with experience of the epidemiology of the two conditions have regarded them as distinct disease entities. But this view is not shared by all specialist bacteriologists, some of whom consider the two organisms to be a single species, with a variant form differing in certain of its biochemical reactions.

Kauffmann (1954) considers that the organisms are best designated by the name *Salmonella gallinarum-pullorum* "as numerous biochemical types of such strains occur, which make an exact distinction between *Salm. gallinarum* and *Salm. pullorum* impossible. . . .Therefore it is proposed to speak of one serological *S. gallinarum-pullorum* type only, which can be divided into different biochemical types, without giving these sub-types special names." Similarly, Topley and Wilson (1955), under the heading of *Salm. gallinarum,* refer to "A more important fermentative variant, often regarded as a separate type and called *Salm. pullorum,* is responsible for the widespread disease of chickens . . . " On the other hand, in Bergey's Manual of Determinative Bacteriology (1948), the two organisms are clearly regarded as separate species.

European investigators have, on the whole, been more inclined to the view that *Salm. pullorum* and *Salm. gallinarum* should be classified as one organism, whereas in America the tendency has been to regard them as two related species causing different diseases. Manninger (1930) and Beaudette (1930) represented these two opinions in papers presented to the same International Veterinary Congress. Hinshaw (1941), after an extensive review of the literature and after examining 454 strains of *Salm. pullorum* and 91 strains of *Salm. gallinarum,* concluded that his studies furnished "additional evidence that *Salmonella pullorum* (Rettger) and *Salmonella gallinarum* (Klein) are separate and distinct species."

At the present time workers in France and Belgium appear to hold different opinions based on investigations carried out in recent years. Lucas, Andral, Bouley, Paraf, and Quinchon (1951) after examining 155 strains of *Salm. pullorum* and 66 strains of *Salm. gallinarum* came to the conclusion that "separation into two species is not supported from the bacteriological point of view." Schoenaers, Kaeckenbeeck and Poor (1955) were in agreement with this opinion, but, on the other hand, Neel, le Minor and Taslimi (1954) and Virat, Vallée and Jacotot (1955) thought it desirable to maintain a distinction between the two organisms.

The amount of diagnostic material received at Weybridge is probably unequalled by any other single laboratory in the United Kingdom and five years ago it was decided to collect further bac-

teriological evidence that pullorum disease and fowl typhoid are two distinct diseases. This paper is an account of the examination and typing of 1007 cultures finally classified as *Salm. pullorum* and 618 cultures classified as *Salm. gallinarum,* all of them isolated from poultry during the period 1950 to 1955. The pullorum cultures were freshly isolated from specimens sent to Weybridge for routine post-mortem examination, but 216 of the *Salm. gallinarum* cultures were subcultures sent to Weybridge for confirmatory typing after isolation by Dr. F. T. W. Jordan, now of Liverpool University, while working at Aberystwyth in Wales on a special study of fowl typhoid.

TECHNIQUE OF EXAMINATION

Routine bacteriological examination in the case of baby chick carcases consisted of primary cultures on MacConkey's agar plates and in peptone broth from the livers and heart blood ; the same procedure was followed in the case of adult fowls with post-mortem lesions indicating a septicaemic disease. Most of the *Salm. pullorum* strains from adult fowls were isolated from healthy birds sent for examination from the field as "reactors" to the routine pullorum blood test; from these specimens the organisms were isolated on desoxycholate citrate agar plates, after one to four days incubation of ground tissues in selenite F enrichment medium. The cultures received from Dr. Jordan were on nutrient agar slopes, having been subcultured after primary isolation. When primary cultures contained a mixed growth, individual non-lactose fermenting colonies were seeded into peptone water and a pure growth obtained by plating on desoxycholate citrate agar plates. Observation of the growth characteristics of colonies of the organisms on solid media played an important part in differentiating *Salm. pullorum* from *Salm. gallinarum.*

The routine carbohydrate media used in this study consisted of one per cent solutions of the carbohydrate in one per cent peptone water containing Andrade's solution as an indicator and a Durham tube to permit recording of gas production; each batch of media was standardised at pH. 7.3.

The following carbohydrates were used :— lactose, maltose, dulcite, sucrose, glucose, mannite, salicin and xylose; in addition peptone water (for indole test), an agar slope (for rapid slide agglutination) and a peptone broth (for tube agglutination tests), were sown at the same time.

Tube agglutination tests were carried out using positive *Salm. pullorum* high titre rabbit serum diluted to give complete agglutination in a dilution of 1:250; in addition, each culture isolated on a nutrient agar slope was examined for evidence of form variation, using absorbed factor sera prepared in rabbits according to the technique described by Wright and Edwards (1948). Rapid slide agglutination tests using pure factor XII_2 and XII_3 sera were carried out with a small amount of each culture, but individual colonies were not examined; this technique seemed justified on the basis of the results presented by Snoeyenbos, Bachman and Van Roekel (1952).

The rapid slide results were checked by tube agglutination tests with absorbed factor sera in the case of the small number of strains judged to be variants i.e. those with virtually no XII_3 agglutination on the slide test. Serological typing of variant cultures examined in 1953 on this basis was

checked by comparison with the sedimentation method described by Williams (1953) and complete agreement was obtained between the two methods.

RESULTS

Morphology

As both *Salm. pullorum* and *Salm. gallinarum* have been described in detail by many authors, it is only intended in this paper to emphasize those characteristics of the organisms which have been found useful in differentiating them.

Salm. gallinarum, when isolated on solid media from fowls dying of acute disease grows more profusely and more vigorously than *Salm. pullorum.* Individual organisms, when examined microscopically, normally appear as shorter and thicker rods, measuring approximately 2.0 x 1.0 μ, as compared with those of *Salm. pullorum* which measure approximately 3.0 x 0.5 μ. The colonies are usually larger and less transparent. Furthermore, a culture of *Salm. gallinarum* produces a striking seminal odour, which is absent from a culture of *Salm. pullorum.* Hendrickson (1927) and Virat, *et. al.* (1955) noted this more vigorous growth of *Salm. gallinarum* and Beaudette (1930), in addition, mentioned the characteristic odour.

Salm. pullorum when isolated from older chickens and chronic "carriers" does not always form the characteristic dew drop colonies; quite frequently, individual colonies are as large as those of *Salm. gallinarum,* but they never give the characteristic odour.

Biochemical Reactions

Salm. pullorum

During 1950, 1951, 1952 and 1954, the main object was to study the organisms isolated from routine outbreaks of pullorum disease in order to be certain that disease was not caused by the variant strain of the organism. During these years, therefore, it was primarily the antigenic structure of the cultures which was under examination. The growth characteristics were noted, but the biochemical reactions were observed in only lactose, maltose and dulcite after 48 hours incubation at 37°C. None of the 419 strains examined during this period fermented lactose or dulcite but five of them fermented maltose. 283 of these strains were isolated from baby chicks, 91 from adult fowls, two from turkey poults and 43 were isolated from unspecified avian sources.

In 1953 and in 1955, 588 cultures were typed, but in addition to examination for antigenic form variation, the fermentation reactions were observed in lactose, maltose, dulcite, sucrose, glucose, mannite, salicin and xylose. The reactions were observed daily for eight days incubation at 37°C.

One of these 588 strains fermented maltose. All fermented glucose and mannite, 104 aerogenically and 484 anaerogenically ; 149 fermented xylose, 94 aerogenically. None produced indole.

Of the 588 strains examined, 445 were isolated from baby chicks, 114 from adult fowls, three from turkey poults, one from a bantam and 25 of them were of avian origin but the species was not recorded.

The presence of only six maltose fermenting strains after eight days incubation in a series of 1097 cultures disagrees with the work of Hinshaw, Browne and Taylor (1943) and Hinshaw, McNeil and Taylor (1944). In the first paper the authors, after examining 300 stock cultures, reported that 43 of 260 anaerogenic strains fermented maltose after 24 hours incubation and another five in 48 hours, while two of 40 anaerogenic strains did so within a week. The second paper described the study of 146 primary cultures isolated from 52 outbreaks of disease in fowls and turkeys in California, which all fermented maltose with gas production in 24 to 72 hours.

The work at Weybridge does not suggest that true maltose fermenting strains are at all common in England and Wales; on the other hand, when 34 of the 1007 strains were incubated continuously for 30 days, 26 of them fermented maltose between 8 and 30 days. If the eight day period had been extended, there is little doubt that a considerable number of slow maltose fermenting strains would have been encountered. The eight day period of incubation was decided upon in this study because it appeared to provide the greatest degree of differentiation between *Salm. pullorum* and *Salm. gallinarum.*

There was no support for the view put forward originally by Hadley, Caldwell, Elkins and Lambert (1917) that aerogenic strains of *Salm. pullorum,* named by them Alpha type, are associated with the chronic "carrier" hen and infection in the egg, whereas the anaerogenic forms, Beta type, are the cause of disease in baby chicks.

Salm. gallinarum

From 1950 to 1955, 618 cultures were typed: 402 strains were isolated from post-mortem specimens at Weybridge and 216 were sent by Dr. Jordan.

The biochemical reactions of 474 of these strains were recorded as for *Salm. pullorum.* Anaerogenic fermentation of glucose, mannite and xylose took place regularly within 24 to 48 hours; 38 strains failed to attack maltose for two to three days and 15 strains were slow to attack dulcite, but all strains fermented maltose and dulcite by the eighth day. None produced indole. The remaining 144 strains were typed as *Salm. gallinarum* on the basis of their growth characteristics, agglutination reactions and fermentation reactions in lactose, maltose and dulcite observed after 48 hours incubation at 37°C.

Of the 618 strains classified as *Salm. gallinarum,* 561 were isolated from adult fowls, 30 from chicks, two from turkey poults, one from an adult turkey and 24 from unspecified avian sources.

At one stage during this work, it was suggested that Simmons citrate agar might be a useful medium for differentiating *Salm. pullorum* from *Salm. gallinarum.* Strains of *Salm. pullorum* (210) were sown on this medium but only one grew after eight days incubation;

strains of *Salm. gallinarum* (58) were treated similarly but only ten of them grew. This medium was therefore not used for subsequent examinations.

Serological Examination

Salm. pullorum

The complete antigenic formula of *Salm. pullorum* is considered to be IX, XII_1, (XII_2), XII_3. Edwards and Bruner (1946) established that antigen XII_2 is variable and that both XII_{2++} and XII_{2+} forms occur; those with XII_3 antigen markedly in excess of XII_2 are regarded as standard strains and those with little or no XII_3 and a large amount of XII_2 are regarded as variants; in addition intermediate strains occur in which both XII_2 and XII_3 antigens are present more or less equally.

Using the technique described earlier 1007 strains of *Salm. pullorum* were examined: 609 were classified as standard strains, 372 were considered intermediate strains and 26 were considered variant strains. It should be noted that this classification was based on the examination of freshly isolated strains. After periods up to 12 months on artificial media some intermediate strains revert to standard form: in addition, a number of the strains classified provisionally as variants sometimes had to be re-classified subsequently as intermediate strains. For example, in 1953, 12 strains were classified as variants on isolation. In eleven of them tests at monthly intervals revealed a rapid development of factor XII_3. In one case there was a loss of XII_2 antigen so that it was finally considered to be a standard strain. Another ten strains subsequently agglutinated almost equally with sera containing both factors XII_2 and XII_3, although the intensity and speed of reaction with XII_2 was always greater than with XII_3 and they were reclassified as intermediate strains. Only one of the original twelve variant strains isolated that year remained consistently negative for factor XII_3.

Salm. gallinarum

Cultures of *Salm. gallinarum* (618) were examined in a similar manner. All of them had a full complement of antigen factor XII_3; only six agglutinated to a detectable degree on the slide test with factor XII_2 serum, but this was never comparable with the amount of agglutination obtained with the XII_3 serum. Tube tests were carried out with these six strains using factor XII_2 serum; five of them were completely negative in serum dilutions as low as 1:10 and one strain agglutinated to a slight degree in the 1:10 dilution. The variant pullorum antigen used as control in these tests agglutinated in serum dilutions up to 1:160. The serological typing of the six strains was checked by the ammonium sulphate sedimentation method of Williams (1953) and all six strains conformed to the standard pattern.

These results, involving the examination of a much greater

number of cultures, support those of Burr (1955) in America who examined from 1948 to 1955, 151 strains of *Salm. pullorum* and 58 strains of *Salm. gallinarum* without finding any evidence of XII_2 antigen in the gallinarum strains. Williams and Harris (1955), in a preliminary report on the sedimentation effect of ammonium sulphate, examined 41 strains of *Salm. gallinarum* and 23 standard strains of *Salm. pullorum*. They found that the gallinarum strains were consistently sedimented by lower concentrations of ammonium sulphate than were those of pullorum. Although the range of sedimentation differences was narrow in contrast to that between standard and variant strains of *Salm. pullorum*, they considered it be distinct and characteristic of each type and on the basis of these results, they suggested that certain minor antigenic differences may exist between *Salm. gallinarum* and standard strains of *Salm. pullorum*.

Several workers, including Kauffmann (1930, 1934), Rodrigues and Pacheco (1936) and Edwards (1939) have attempted unsuccessfully to separate the two organisms on the basis of agglutination reactions and agglutinin-absorption tests, but the possibility of there being hitherto undetected antigenic differences between these organisms is certainly suggested by the results of the serological examination of the cultures described in this paper. The virtual absence of form variation in the *Salm. gallinarum* cultures was striking. It remains true however, that *Salm. gallinarum* is indistinguishable by this technique from standard strains of *Salm. pullorum*.

DISCUSSION

The work reported in this paper was carried out in a busy diagnostic laboratory and it was not the intention to make a detailed bacteriological study of these cultures. It is well known that some organisms alter quite markedly both morphologically and in their biochemical properties after periods of artificial cultivation and storage, and it is suggested that inconsistencies which emerge after a considerable period of time on or in artificial media should be ignored when considering the case for regarding *Salm. pullorum* and *Salm. gallinarum* as distinct species.

The growth characteristics and odour of the *Salm. gallinarum* cultures, the virtual absence of antigen factor XII_2 and the rapid fermentation of dulcite are three constant features of this organism. The case for regarding *Salm pullorum.* and *Salm. gallinarum* as two distinct species is considerably strengthened by the demonstration of small antigenic differences between some strains of pullorum and those of gallinarum. The apparent antigenic identity has been one of the main difficulties facing those who, on other grounds, were prepared to regard the two organisms as separate species. The serological technique used in this paper would not distinguish between a standard strain of *Salm. pullorum* and *Salm. gallinarum*, but the demonstration of form variation in one species and its absence in the other is itself considered significant.

The presence of only six maltose fermenting strains of *Salm. pullorum* among 1007 cultures (588 of them observed for eight days and 419 of them for two days) does not seem of much significance although it is to some extent at variance with the work of Hinshaw, *et al.* (1943, 1944); the fermentation of maltose together with dulcite within 48 hours can be taken as strongly indicative of *Salm. gallinarum*. The reaction of *Salm. gallinarum* in cysteine gelatin as reported by Hinshaw (1941) provides yet another characteristic by which this organism can be differentiated from *Salm. pullorum*. He found that the 454 pullorum strains examined at that time were negative in this medium, whereas all but two of his 91 strains of *Salm. gallinarum* produced a characteristic reaction. This differential medium, however, was not used in the studies reported in this paper.

A discussion of the differential characteristics of two organisms would be incomplete without reference to the diseases they produce. In general terms, it would be true to state that *Salm. pullorum* causes an acute epidemic disease in baby chicks commonly in the fowl and more rarely in other species; whereas *Salm. gallinarum* produces an epidemic disease in the growing chicken or adult fowl and occasionally in other species. In America however, severe outbreaks of fowl typhoid are a frequent occurrence in turkeys. During the past 12 years only two outbreaks of acute disease caused by *Salm. pullorum* in adult fowls have been diagnosed at Weybridge and these were both attributed to the feeding of inadequately sterilized poultry offal. Until quite recently, mortality among baby chicks in Great Britain due to *Salm. gallinarum* infection has been very uncommon. During the three years 1950 to 1952, only one outbreak of fowl typhoid was diagnosed at Weybridge in baby chicks; in 1953, 1954 and 1955, six, ten and thirteen outbreaks respectively were diagnosed, the increased numbers diagnosed in 1954 being due, in part, to outbreaks occurring in chicks supplied through a single hatchery. In 1954, there was an outbreak in turkey poults and in 1955 another two in this species.

The majority of the 205 strains of *Salm. pullorum* from adult fowls were isolated during the bacteriological examination of healthy "carriers" of pullorum disease detected in the field by the agglutination test; a few were recovered from individual dead fowls sent for routine post-mortem examination, most of which had died from other causes. None of the pullorum strains was isolated from adult fowls dying from acute pullorum disease.

On epidemiological grounds alone, it seems incorrect to regard *Salm. pullorum* as merely "a more important fermentative variant" of *Salm. gallinarum*. The results reported in this paper on the examination of 1007 cultures of *Salm. pullorum* and 618 cultures of *Salm. gallinarum* are considered to provide overwhelming evidence, particularly when considered alongside the earlier work of Hinshaw and others, that *Salm. pullorum* and *Salm. gallinarum* are separate and distinct species in spite of their close antigenic relationship.

CONCLUSIONS

An account is given of the study of 1007 cultures of *Salm. pullorum* and 618 cultures of *Salm. gallinarum* isolated from poultry during the years 1950 to 1955.

It is considered that the results taken in conjunction with the work of earlier investigators, provide conclusive evidence, that *Salm. pullorum* and *Salm. gallinarum* are separate and distinct species.

The differentiation of the two species is based on their growth characteristics and biochemical reactions, with particular reference to the fermentation of maltose and dulcite, and on the absence of antigenic form variation in cultures of *Salm. gallinarum*.

It is further considered that these differences between the two organisms must be related to the distinctive epidemiology of acute outbreaks of pullorum disease and fowl typhoid as occurring in the field.

REFERENCES

Beaudette, F. R. (1930). *Proc. 11th Internat. vet. Cong.*, **3**, 705.

Bergey (1948). *Manual of Determinative Bacteriology*. 6th Edit. pp. 520-521. Williams and Wilkins Co.; Baltimore.

Burr, W. E. (1955). *Proc. 27th Ann. meet. N.E. Conf. Lab. workers on Pull. dis. control.* Poult Dept. Univ. New Hampshire.

Edwards, P. R. (1939). *Kentucky agric. Exp. Sta. Circ.*, No. 50.

Edwards, P. R., and Bruner, D. W. (1946). *Cornell Vet.*, **36**, 318.

Hadley, P., Caldwell, D. W., Elkins, M. W., and Lambert, D. J. (1917). *Bull. Rhode Island agric. Exp. Stat.* No. 172.

Hendrickson, J. M. (1927). *J. Amer. vet. med. Assoc.*, **70**, 629.

Hinshaw, W. R. (1941). *Hilgardia*, **13**, 583.

Hinshaw, W. R., Browne, A. S., and Taylor, T. J. (1953). *J. infect. Dis.*, **72**, 197.

Hinshaw, W. R., McNeil, E., and Taylor, T. J. (1944). *Poult. Sci.*, **23**, 94.

Kauffmann, F. (1930). *Zbl. Bakt. 1 Orig.*, **119**, 152; (1934). *Ibid*, **132**, 337; (1954). *Enterobacteriaceae*, 2nd Edit. p. 45. Munksgaard; Copenhagen.

Klein, E. (1889). *Zbl. Bakt.*, **5**, 689.

Lucas, A., Andral L., Bouley, G., Paraf, A., and Quinchon, C. (1951). *Rec. Méd. vet.*, **127**, 547.

Manninger, R. (1930). *Proc. 11th Internat. vet. Cong.*, **3**, 724.

Neel, R., Le Minor, L., and Taslimi, H. (1954). *Ann Inst. Pasteur*, **87**, 719.

Rettger, L. F. (1900). *N.Y. med. Jour.*, **71**, 803.

Rodrigues, C., and Pacheco, G. (1936). *Compt. rend. Soc. Biol.* Paris, **123**, 438.

Schoenaers, F., Kaeckenbeeck, A., and Poor, H. S. (1955). *Ann. méd. Vet.*, **1**, 40.

Snoeyenbos, G. H., Bachman, B. A., and Van Roekel, H. (1952). *Poult. Sci.*, **31**, 1009.

Topley and Wilson. (1955). *Principles of Bacteriology and Immunity*, 4th edit. **1**, 831. Edward Arnold; London.

Virat, B., Vallée, A., and Jacatot, H. (1955). *Ann Inst. Pasteur*, **88**, 133.

Williams, J. E. (1953). *Amer. J. vet. Res.* **14**, 458 and 465.

Williams, J. E., and Harris, M. E. (1955). *Proc. 27th Ann. meet. N.E. Conf. Lab. workers on Pull. Dis. Control.* Poultry Dept. Univ., New Hampshire.

Wright, M. L., and Edwards, P.R. (1948). *Amer. J. vet. Res.*, **9**, 386.

[Received for publication, January 16th, 1956]

THE EFFECT OF TICK-BORNE FEVER ON THE RESISTANCE OF LAMBS TO STAPHYLOCOCCI

By

A. Foggie

Moredun Institute, Gilmerton, Edinburgh

INTRODUCTION

Although tick pyaemia of lambs is constantly associated with tick infestation, the rôle of the tick in the pathogenesis of the disease is still unknown. Previous experiments (Foggie, 1947) showed that the tick is unlikely to act as a vector of the staphylococcus. As there appears to be no correlation between the morbidity from tick pyaemia and the numbers of ticks on different pastures the possibility that the predisposing factor to tick pyaemia is some other disease of which the tick is a true vector, requires further study. It has been shown (McEwen, 1947) that the majority of lambs on a tick-infested pasture become infected with tick-borne fever in the first few weeks of life and it appeared possible that this infection might lower the lamb's resistance to infection with staphylococci. Taylor, Holman and Gordon (1941) recognised this possibility but failed to produce pyaemia by the subcutaneous injection of staphylococci into lambs during the febrile stage of an attack of tick-borne fever. The animals in their experiment were, however, three to four months old, i.e., over the age at which the disease is encountered in the field.

In the present experiments the effects on young lambs of tick-borne fever and the intravenous injection of staphylococci have been examined.

METHODS

Animals. The lambs used in the experiment were the progeny of ewes maintained at the Institute during gestation and lambing. They were less than four weeks old when given the first inoculation. When twins were available one was used as a control to the other. Single lambs were paired as evenly as possible according to size. The largest lambs were given the largest infective dose of staphylococci and vice-versa.

Tick-borne fever strain. The same strain of tick-borne fever was used throughout, the infection being transmitted by the intravenous inoculation of blood from an infected sheep.

Staphylococcus. A strain of an αβ haemolytic staphylococcus (1468), which had been retained in the dry state for several years, was used in these experiments. Infective doses were prepared by washing the growth off an 18 hour agar culture with saline, standardising the suspension to No. 8 of Brown's opacity tubes and making the required dilutions of this suspension. A volume of 1cc. of these dilutions was used as the infective dose.

Blood counts. Total white cell counts and differential counts were made from blood withdrawn from the jugular vein.

Blood cultures. Sheep-blood agar slopes were prepared in 1 oz. medicine flats. After the agar had set, 3 cc. of broth were added to each flat and a sterile pierceable rubber cap was fitted. A sample of approximately 1 cc. of blood was withdrawn, with aseptic precautions, from the left jugular vein and injected directly through the rubber cap into the broth. The mixture of blood and broth was flooded over the surface of the agar and the flat was incubated in the vertical position. If no colonies were present on the agar after 24 hours the broth was again flooded over the agar and incubation was repeated. In this way it was hoped to obtain a comparative colony count if infection was heavy and to isolate staphylococci from the broth culture if infection was light.

Antitoxin content of sera. Sera from the lambs in the experiments were tested for the presence of antibody to α staphylococcal toxin by the haemolysis test, the result being expressed in International Standard Units.

Post-mortem examinations. Post-mortem examinations were made on all the lambs and, at each autopsy, material from liver, lung, heart, spleen and kidney, and also from any macroscopic lesion, was sown on sheep-blood agar plates. A diagnosis of pyaemia was made when multiple abscesses were present.

EXPERIMENTAL FINDINGS

Tick-borne Fever Infection

In a preliminary experiment, a nine-day old lamb (1) was infected with tick-borne fever and daily temperatures and neutrophil counts were taken. The temperature started to rise five days after inoculation and reached its maximum of 107°F. on the ninth day; thereafter it dropped to a low normal level. The neutrophil count fluctuated between 1000 and 3500 cells per c.mm. until the ninth day after infection when it dropped below 800 and remained below this level for a further seven days, reaching its minimum of 160 on the 12th

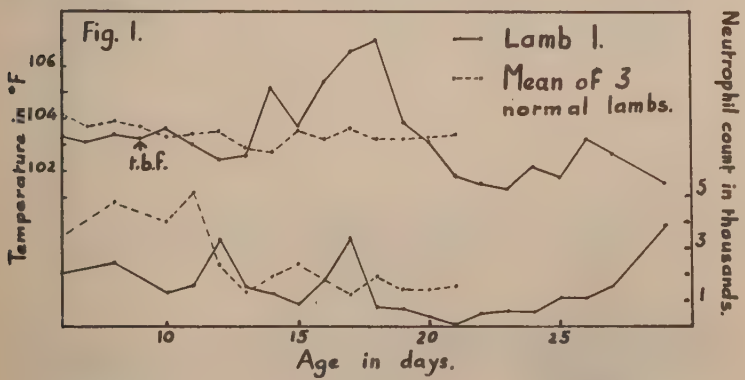


Fig. 1. t.b.f.=inoculated with tick-borne fever blood. The upper curves relate to temperature: the lower to neutrophil counts.

day after infection. These findings are illustrated in Fig. 1 where they are compared with mean temperatures and mean neutrophil counts of three normal lambs of the same age.

Delayed Infection Tick-borne Fever and Staphylococci

In the first staphylococcal infection experiment one lamb in each of three pairs of lambs (2 to 7) was infected with tick-borne fever and when the neutrophil count dropped below 800 cells per c.mm. of blood this lamb and its control were inoculated intravenously with

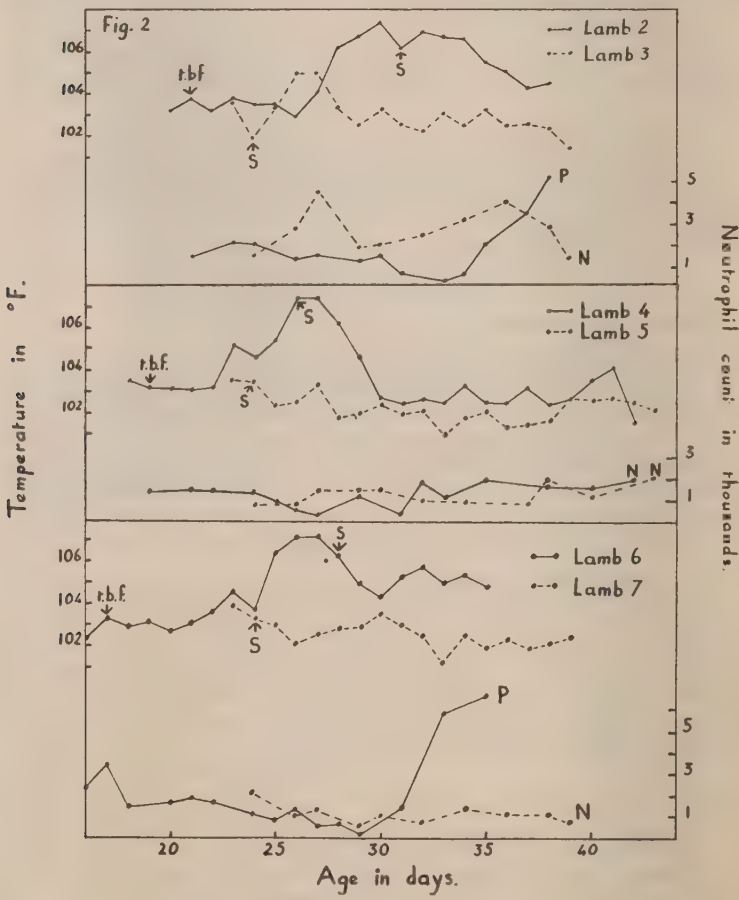


Fig. 2. S.=inoculated with staphylococci.
Lambs 2 & 3 received 1/1000 of a No. 8 B.S. staphylococcal suspension.
Lambs 4 & 5 „ 1/10000 „ „ „ „ „ „ „ „
Lambs 6 & 7 „ 1/50000 „ „ „ „ „ „ „ „
P= staphylococcal abscesses present at post-mortem examination.
N=no abscesses present at post-mortem examination.

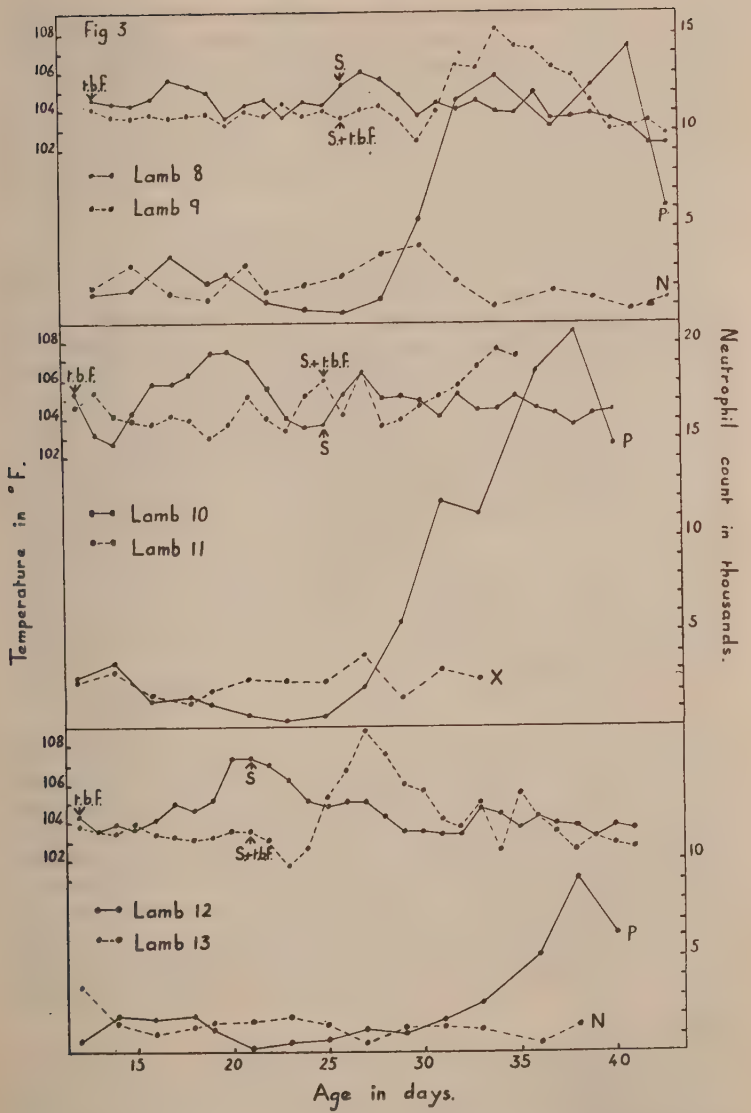


Fig. 3. S. + t.b.f. = inoculated simultaneously with staphylococci and tick-borne fever blood.
Lambs 8 & 9 received 1/1000 of a No. 8 B.S. staphylococcal suspension.
Lambs 10 & 11 „ 1/10000 „ „ „ „ „ „ „ „
Lambs 12 & 13 „ 1/50000 „ „ „ „ „ „ „ „
X = lamb died of an intercurrent pasteurella infection.

the same dilution of staphylococcal suspension. Each pair of lambs received a different dilution of the suspension, namely, 1:1000, 1:10,000 and 1:50,000. The tick-borne fever infected and neutropaenic lambs inoculated respectively with dilutions 1:1000 and 1:50,000 developed pyaemia. Pyaemia was not diagnosed in the neutropaenic lamb which received the 1:10,000 dilution nor in the three control lambs. The temperatures and neutrophil counts of these lambs are shown in Fig. 2.

Simultaneous Infection Tick-borne Fever and Staphylococci

A further six lambs (8 to 13) were used to test the effect of the simultaneous injection of staphylococci and tick-borne fever. The same procedure as in the previous experiment was adopted, but in addition when the lambs were inoculated with staphylococci the hitherto normal control lambs were at the same time inoculated with tick-borne fever blood. The three lambs suffering from a tick-borne fever neutropaenia at the time of infection with staphylococci all developed pyaemia. Pyaemia did not however develop in any of the three lambs inoculated simultaneously with staphylococci and tick-borne fever blood, although all suffered from an attack of tick-borne fever and one (11) died from an intercurrent pasteurellosis. Cultures from the organs of this animal showed no staphylococci. The lowering of the resistance of sheep to Pasteurella infection by a previous attack of tick-borne fever has already been reported, (Foggie, 1951). The temperatures and neutrophil counts of these lambs are shown graphically in Fig. 3.

The results of the above experiments are summarised in Table I.

Post-mortem Findings

With the exception of lamb 11 all the lambs were killed within 21 days of inoculation with staphylococci. Multiple abscesses were present in the five lambs in which pyaemia was recorded. The most severely affected (2) had abscesses in both carpal, both fore and one hind fetlock, and the left hock joints, three costochondral junctions, the diaphragm, the spleen and one kidney, while the least affected (6) had abscesses in a costochondral junction, one kidney and one lung. Staphylococci showing αβ haemolysis were isolated from all five pyaemic lambs. Abscesses were not found in the remaining six lambs and staphylococci were not isolated on culture of the organs.

Neutrophil Counts

At the time of inoculation with staphylococci the six lambs affected with tick-borne fever had neutrophil counts of between 187 and 641 cells per c.mm. of blood, while five of the six normal lambs when given the staphylococci had counts between 1465 and 2355 cells per c.mm. (Table 1). In the sixth normal lamb (5) there were 738 neutrophils. The counts remained low throughout the experiment, but the eosinophil counts were high, being either just below or occasionally exceeding the neutrophil count. Clinical pyaemia was

TABLE 1

Lamb No.	Age in days at inoculation with tick-borne fever	Dilution of No. 8 B.S. staph. suspension inoculated	Age in days at inoculation of staphylococci	Neutrophil count at inoculation. Cells per c.mm. of blood	Time of death in days after staph. inoculation	Post-mortem findings	Antitoxic titre in International standard units per c.c. serum	
							Before staph. inoculation	At autopsy
				Experiment 1				
2	21	1/1000	31	583	7	Pyaemia	<1	<1
3	—	,,	24	1465	15	No lesions		<1
4	19	1/10000	26	517	16	No lesions	<1	4
5	—	,,	24	738	19	No lesions		<1
6	17	1/50000	28	641	7	Pyaemia	<1	<1
7	—	,,	24	2101	15	No lesions		<1
				Experiment 2				
8	13	1/1000	26	535	18	Pyaemia	<1	350
9	26	,,	26	2535	18	No lesions	1	1
10	12	1/10000	25	610	15	Pyaemia	<1	100
11 T	25	,,	25	2321	11	Pasteurellosis	<1	<1
12	12	1/50000	21	187	20	Pyaemia	<1	10
13 T	21	,,	21	1639	20	No lesions	<1	1

T=twins.

accompanied by an increase in the number of circulating neutrophils (Figs. 2 and 3). The degree of this increase appeared to be correlated with the number and size of the abscesses produced.

Antitoxic Content of Sera

Prior to infection with staphylococci, sera from nine of the lambs were tested for antibody to the α staphylococcal haemolysin and all had titres of less than 1 unit per cc. Sera from all the lambs were tested again before death. Of the five lambs which showed pyaemia three (8, 10 and 12) had titres of 350, 100 and 10 units; the two remaining lambs, (2 and 6) were slaughtered 7 days after infection with staphylococci and in them the titre was still less than 1 unit. None of the lambs, which failed to develop pyaemia, had titres greater than 1 unit at the time of slaughter with the exception of No. 4 which had a titre of 4 units. This lamb had been infected with a 1/10,000 dilution of the staphylococcal suspension during the neutropaenic phase of a tick-borne fever attack and, although it failed to develop pyaemia, it may have had a focus of staphylococcal infection that was inapparent at post-mortem examination.

Blood Cultures

Starting 24 hours after the inoculation of staphylococci, daily blood cultures were made from lambs 8 to 13. In spite of the fact that 8, 10 and 12 developed pyaemia all these cultures were negative suggesting that following intravenous inoculation the bacteraemic phase of the infection is transient.

DISCUSSION

These experiments show that lambs suffering from a neutropaenia resulting from an attack of tick-borne fever are more susceptible to a blood-borne staphylococcal infection than normal lambs. In lambs infected simultaneously with staphylococci and tick-borne fever no increased susceptibility to the bacterial infection was demonstrated, presumably because the staphylococci were overcome before the lambs' resistance had been reduced by the tick-borne fever reaction. Of the two measurable factors in the tick-borne fever reaction, namely fever and neutropaenia, the latter appears to be the more important in reducing the lambs' resistance to staphylococci. For example lamb 10 (Fig. 3) developed pyaemia although it was inoculated with staphylococci two days after the temperature had returned to normal.

According to Gordon, Brownlee, Wilson and MacLeod (1932) the incubation period of tick-borne fever following tick infestation is 6 to 11 days so that a lamb, bitten by infected ticks in the first week of life, would be in the neutropaenic phase of the resultant attack of tick-borne fever when it was between two and four weeks old. At this age any resistance derived from humoral antibody is also low. Earlier work (Foggie, 1948) showed that staphylococcal antitoxin derived

from the colostrum disappeared from the lambs serum by about the 20th day of life and that it was difficult to stimulate an active immunity in lambs under 4 to 6 weeks old. This period coincides with the age at which tick pyaemia cases occur in the field. The mean age at first appearance of clinical signs of 54 naturally occurring cases was 3.8 weeks ± 1 week (Foggie, 1948). It seems probable therefore that the neutropaenia resulting from tick-borne fever at a time when the lamb is low in protective antibody is the main predisposing factor in the disease.

CONCLUSIONS

Five out of six lambs infected intravenously with staphylococci during the neutropaenic phase of an attack of tick-borne fever developed pyaemia.

Three lambs infected with staphylococci only failed to develop pyaemia.

Three lambs infected simultaneously with staphylococci and tick-borne fever failed to develop pyaemia: one of these lambs died from acute Pasteurellosis.

The relationship between tick-borne fever and tick pyaemia is discussed.

REFERENCES

Foggie, A. (1947). *J. comp. Path.*, **57**, 245; (1948). *Ibid.*, **58**, 24; (1951). *J. Path. Bact.*, **63**, 1.

Gordon, W. S., Brownlee, A., Wilson, D. R., and MacLeod, J. (1932). *J. comp. Path.*, **45**, 106.

McEwen, A. D. (1947). *Vet. Rec.*, **59**, 198.

Taylor, A. W., Holman, H. H., and Gordon, W. S. (1941). *Ibid.*, **53**, 337.

[*Received for publication, January 26th, 1956*]

THE MATERNAL TRANSFERENCE OF ANTIBODIES IN THE BOVINE

By

F. Blakemore* and R. J. Garner

Veterinary Laboratory, University of Bristol

INTRODUCTION

Woodman and Hammond (1922, 1923) and Asdell (1925) showed that at about the fifth month of pregnancy the udder of the primiparous bovine begins to secrete a fluid, the precolostrum, rich in globulin. Blakemore (1951) was able to demonstrate that the agglutinin titre of the precolostrum of heifers immunized against *Br. abortus* was always high and often thirty times greater than that of the blood.

As the physical nature of precolostrum restricts the use of conventional immunological techniques, the possibility was explored of following the transfer from blood to precolostrum of antibodies trace-labelled with radio-iodine (^{131}I). This isotope was chosen as being readily available, easy to measure and having a conveniently long half-life (8·1 days) for short-term in vivo studies.

METHODS

As a pregnant heifer was not available at the time this pilot experiment was carried out, a cow, due to produce her third calf in approximately three weeks, was selected.

Preparation of iodinated γ-globulin. The γ-globulins from approximately 600 ml. of serum were separated by the method of Keckwick (1940) and iodinated to the extent of 0·9 per cent by the procedure described by Francis, Mulligan and Wormall (1951) using 5 mc. ^{131}I (as $Na^{131}I$). The final solution (85 ml.), after dialysis and centrifugation, contained 5·6 g. of protein (as measured by micro-Kjeldahl total-N determination), and was shown to be homogeneous by paper electrophoresis; it was sterilized by filtration (Seitz S.G.).

Experimental procedure. The left-hand fore-quarter of the udder had been stripped on each of the two days previous to the commencement of the experiment. Immediately before injection of the solution of labelled protein the secretion from this quarter was again removed as completely as possible and the time at completion of this operation designated zero. The mid-time of intravenous injection of the γ-globulin solution was zero plus 5 minutes. Blood samples were taken from the opposite jugular vein 10, 20, 35, 105, 145, 185, 230 and 275 minutes after the injection and thereafter twice daily (at approximately 9 a.m. and 3 p.m.) for the next 9 days. The chosen fore-quarter was stripped at zero plus 115 and 250

*Deceased.

minutes and thereafter daily (at approximately 12 noon) for the next 9 days, the volume of secretion being noted.

Radioactivity determinations. All counting was carried out using an M.6 liquid counter (Twentieth Century Electronics Ltd.) with a D.4100/1 scaling unit fed through a D.4019/Q quench-type pre-amplifier (Labgear, Cambridge Ltd.). Plasma determinations were performed on 2 ml. aliquots diluted to 10 ml. with 0·02 N-NaOH. Precolostrum samples were treated in the same way using either the whole of the secretion, if the volume was less than 2 ml., or a 2 ml. aliquot.

RESULTS

The results obtained are illustrated in Fig. 1 which gives the corrected activity in counts per minute per ml. of plasma or mammary gland secretion and the volume of the latter.

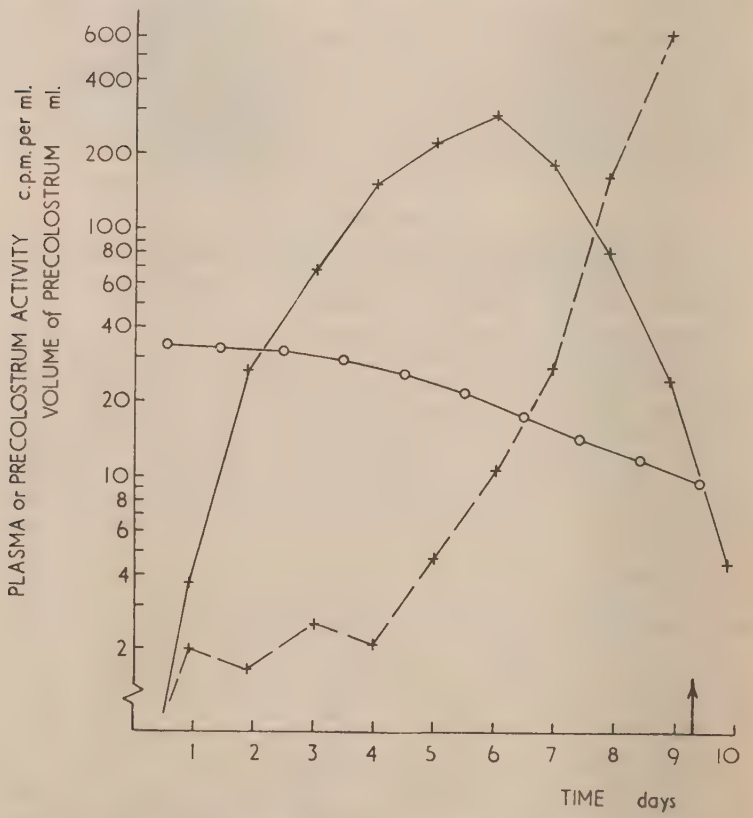


Fig. 1. Graph showing plasma activity (o——o), precolostrum activity (×——×) and volume of precolostrum (× – – – ×) during the course of the experiment. The cow calved at the point indicated by the arrow on the time scale.

Although some activity was detectable in the 115 minute precolostrum sample, significant activity did not appear until 22 hours after injection of the labelled γ-globulins. A maximum was reached on the fifth day of the experiment when the mammary gland secretion possessed approximately 13 times the activity of the plasma.

On the 7th day after injection the secretion had begun to increase rapidly in amount and had taken on the appearance of colostrum; at the same time its activity declined rapidly. The cow calved on the 9th day.

DISCUSSION

Miller and Weetch (1955) showed that the concentration of inorganic iodide containing ^{131}I is considerably greater in breast-milk than in plasma obtained at the same time. If iodination is carried out as described above, iodine appears to be very firmly bound to the protein molecule and in the present instance it was shown that 97 per cent. of the activity of the plasma and 96 per cent. of that of the precolostrum resided in the protein fraction. The early samples of precolostrum (up to the 5th day) contained only γ-globulin by paper electrophoresis. It has thus been shown unequivocally that maternal γ-globulins appear in the precolostrum and are concentrated by the mammary gland in the multiparous as well as the primiparous bovine. Francis, *et al.* (1955) have demonstrated that the immunological behaviour of antibodies is not affected by iodination provided the degree of iodination is kept below 1 per cent. It is therefore reasonable to assume that the precolostral γ-globulins contained unaltered maternal antibodies.

In this pilot experiment no attempt was made to determine the extent to which specific antibodies are secreted and concentrated. It may be possible to extend these observations by combining radio-isotope methods with immunological techniques and by employing more sensitive counting methods.

CONCLUSIONS

Homologous bovine γ-globulins, and presumably antibodies, labelled with ^{131}I appear in the precolostrum within 22 hours of their introduction into the blood-stream and are concentrated by the mammary gland at least 13 times.

REFERENCES

Asdell, S. A. (1925). *J. agric. Sci.*, **15**, 358.
Blakemore, F. (1951). *Vet. Rec.*, **63**, 697.
Francis, G. E., Mulligan, W., and Wormall, A. (1952). *Nature, Lond.*, **167**, 748; (1955). *Biochem. J.*, **60**, 363.
Keckwick, R. A. (1940). *Ibid.*, **34**, 1248.
Miller, H., and Weetch, R. S. (1955). *Lancet, ii*, 1013.
Woodman, A., and Hammond, J. (1922). *J. agric. Sci.*, **12**, 197; (1923). *Ibid.*, **13**, 180.

[*Received for publication, February 14th,* 1956]

THE USE OF FIBROMA VIRUS (SHOPE) FOR THE PROTECTION OF RABBITS AGAINST MYXOMATOSIS

By

Barbara Rowe, W. Mansi and J. R. Hudson

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

Shope (1936, 1938) showed that the inoculation of fibroma virus conferred a high degree of protection to rabbits against experimental inoculation with myxoma virus. He reported that fifty-nine out of sixty-two of his fibroma-treated rabbits resisted challenge by inoculation with the latter. The early results of Shope were confirmed by Berry and Litchty (1936), Martin (1936), Hurst (1938), Hyde (1939), Jacotot and Vallée (1953), and Fenner and Woodroofe (1954), and a brief summary of early work performed at Weybridge was given by Ritchie, Hudson and Thompson (1954). The percentage of rabbits that have been found to have acquired a serviceable immunity after inoculation with fibroma virus has varied from one worker to another.

The present paper gives a detailed account of our investigation of the keeping properties of the vaccine, the duration of immunity, the safety of the vaccine for young rabbits, the effect of immunity of the dam on the result of vaccinating suckling rabbits and the relative value of the two sub-strains of the Boerlage strain of fibroma virus isolated by Shope in 1947, given subcutaneously and intradermally.

MATERIALS AND METHODS

Rabbits. Seven hundred and ninety-nine rabbits were used in these experiments. Some were purchased in the open market, some were bred, but none had been immunized before being put on an experiment. They varied in age and breed.

Fibroma virus (Shope). Two sub-strains of the Boerlage strain of this virus were used. The first, obtained from Dr. Jacotot in September, 1953, is referred to as "P", and the second, from Professor Fenner, is referred to as "F". Vaccine was normally prepared by the method used at the Pasteur Institute, Paris. Male rabbits were inoculated into each testicle and subcutaneously at four or more sites with 0·5 cc. doses of a ten per cent suspension of material harvested previously. The seed virus came from a bank, stored frozen at —40°C., and, in order to minimise the possibility of changes in the virus, no unnecessary passages were made.

On the day before the maximum development of the tumours was expected, usually on the eighth day, the rabbits were killed and the testicles and subcutaneous, oedematous pulp were collected, weighed and homogenized in a "Magimix" household pulping machine in five volumes of five per cent whole-egg buffer of pH 7·4. This concentrated suspension was titrated on rabbits and stored frozen until required. The vaccine itself was then prepared by diluting the concentrate so that each 0·5 cc. dose contained 1,000 m.i.d. Usually, this meant diluting 10 to 100 fold.

Myxoma virus. A fully virulent strain, isolated from a rabbit from Cornwall in April, 1954, was used. The material was kept frozen during the early part of the work, but in September, 1954, freeze-dried material was prepared and, since then, challenge has been mainly with this material. Inoculation of this strain produced typical myxomatosis in susceptible rabbits, with death in 11 to 15 days. The preparation before freeze-drying contained between 10^{-6} and 10^{-10} m.i.d. per cc. The challenge dose was at least 1,000 m.i.d. and was inoculated intradermally or subcutaneously.

RESULTS

Keeping Properties of the Vaccine Concentrate

A number of rabbits were inoculated with the original material from Paris and the virus thus obtained has been used to infect all the vaccine producers required in our work. Retention of the concentrated vaccine for several months made it necessary to determine the period during which it could be kept without loss of activity. Table 1 shows that when stored frozen at —40°C. the

TABLE 1

KEEPING PROPERTIES OF THE VACCINE CONCENTRATE (1/5)

Temperature	*Period in weeks*	*Diluent*	*Numbers inoculated*	*Fibroma reaction*	*Myxoma challenge*		
					I.	*R.*	*D.*
—40°C.	1	Buffer saline pH 7·4	13	12	11	0	2
	2	,, ,, ,, ,,	3	3	1	1	1
	9	,, ,, ,, ,,	5	5	2	2	1
	15	,, ,, ,, ,,	43	11	29	9	5
	32	,, ,, ,, ,,	3	2	1	2	0
	36	,, ,, ,, ,,	5	2	2	2	1
	46	,, ,, ,, ,,	3	3	2	1	0
	52	,, ,, ,, ,,	3	0	3	0	0
+4°C.	1	Beef-heart infusion broth pH 7·4 and freeze-dried	4	4	4	0	0
	2	,, ,,	3	2	3	0	0
	39	,, ,,	3	3	3	0	0
	58		3	3	2	1	0
+4°C.	69	Buffer saline pH 7·4 and freeze-dried	2	2	1	1	0
	88		3	3	2	0	1

I=Immune. R=Recovered. D=Died.

concentrate can be held for a year. After freeze-drying it may be kept at —4°C. for a longer period, both in buffer saline or in beef-heart infusion broth. Tests after longer periods of storage have still to be made.

Keeping Properties of the Diluted Vaccine

A series of preparations were made from one batch of concentrate (sub-strain P) by diluting with the following:— (a) phosphate

buffer saline pH 7·4; (b) phosphate buffer saline pH 7·4 containing five per cent whole egg; (c) phosphate buffer with 1:100,000 thiomersalate. Part of the preparation containing egg, but without thiomersalate was freeze-dried. Samples of the liquid preparations were stored at room temperature and at +4°C. Freeze-dried material was stored at +4°C. and reconstituted to the original volume immediately before use. The results of tests of these samples are given in Table 2. It will be observed that neither the addition

TABLE 2

KEEPING PROPERTIES OF FIBROMA VIRUS SUSPENSIONS OF VACCINE STRENGTH

Period of storage in weeks	*Preparation and temperature of storage*	*Number of rabbits*				
		Inoculated	*Reacted*	*Results of challenge*		
				Immune	*Recovered*	*Died*
1	Buffer saline pH 7·4 +4°C.	2	2	3	—	—
	Egg buffer +4°C.	2	1	1	—	1
	Egg buffer + thiomersalate +4°C.	3	2	2	—	—
2	Buffer saline pH 7·4 +4°C.	7	6	5	1	1
	Egg buffer +4°C.	3	2	3	—	—
	Egg buffer + thiomersalate +4°C.	3	2	1	2	—
20	Buffer saline pH 7·4 +4°C.	4	—	—	—	4
	Egg buffer +4°C.	4	—	—	—	4
	Egg buffer + thiomersalate +4°C.	4	—	—	—	4
	Egg buffer Room temperature	2	—	—	—	2
	Egg buffer + thiomersalate „	4	—	—	—	4
20	Egg buffer, freeze-dried +4°C.	3	3	—	2	1
32	„ „ „ „ +4°C.	3	3	—	3	—
41	„ „ „ „ +4°C.	3	3	1	1	1
94	„ „ „ „ +4°C.	3	1	—	—	3

of egg proteins as protective colloids nor thiomersalate as a bacteriostatic agent improved the keeping qualities of the vaccine so that it gave any protection after storage for five months in the refrigerator. Freeze-drying enabled the vaccine to be kept for periods up to twenty-two months, but the extra cost entailed in producing a freeze-dried vaccine would perhaps render such a product uneconomic.

Duration of Immunity

The rabbits included in Table 3 were inoculated with different batches of vaccine at various times, but the vaccine was always standardized to contain about 1,000 minimum infective doses of fibroma virus and it was always used fresh. It is felt that this table gives a general picture of what may be expected of the vaccine and it is probably a better guide to the value of the vaccine in the field

than an experiment designed with one batch of vaccine on a single large group of rabbits. It will be observed that about three quarters of the rabbits vaccinated had a serviceable immunity for about a year.

TABLE 3

DURATION OF IMMUNITY

Period between vaccination and challenge	*Number of rabbits vaccinated*	*Result of challenge*		
		Immune	*Recovered*	*Died*
Under 60 days	34	29 (86%)	5 (14%)	0 (%)
Between 60–120 days ..	41	24 (60%)	11 (28%)	6 (12%)
Between 120–200 days ..	67	24 (36%)	26 (39%)	17 (25%)
Between 200–300 days ..	36	26 (73%)	4 (11%)	6 (16%)
Between 300–400 days ..	12	6 (50%)	3 (25%)	3 (25%)
Between 400–500 days ..	14	3 (22%)	1 (7%)	10 (71%)
Over 500 days	4	0 (0%)	1 (25%)	3 (75%)

Safety and Value of the Vaccine in Baby Rabbits

The following experiments were designed to find out how young, suckling rabbits could be vaccinated safely and whether the immunity of the dam interfered with the result of vaccination.

Many litters of rabbits were used in these experiments and several different batches of freshly-prepared vaccine. The results are given in Table 4 in which are also included the results of tests made with myxoma virus. It will be observed that during the first three weeks of life the offspring of fibroma-immune rabbits are less likely to react to a dose of fibroma virus than are the offspring of a susceptible rabbit. Contrary to what is found with fibroma virus, it is clear that no protection is afforded to the young of myxoma-immune does against inoculation with myxoma virus. This result confirms those reported by Jacotot, Vallée and Virat (1954b).

Unfortunately, a proportion of the young rabbits was lost before the second part of this experiment, an attempt to study the possibility that the young of a vaccinated dam might not respond as well as those of a susceptible dam to vaccination, could be completed. Nevertheless, it was possible to challenge 81 offspring from vaccinated does, themselves vaccinated when two to seven weeks old, and 14 offspring from normal does, vaccinated when four to seven weeks old. The results (Table 5) suggest that the immunity of the doe does not affect the development of immunity in the young.

Effect of Vaccination on Pregnant Does

Pregnant does did not abort as a result of the vaccination, but we observed some abortions in pregnant rabbits which were in-

TABLE 4

SUSCEPTIBILITY TO EXPERIMENTAL INFECTION WITH FIBROMA AND MYXOMA VIRUSES

Offspring of myxoma immune, fibroma immune and normal rabbits.

Virus inoculated	*Age in weeks when inoculated*	*Offspring of myxoma-immune rabbits*				*Offspring of fibroma-immune rabbits*				*Offspring of normal rabbits*			
		Number inoculated	*No reaction*	*Reacted*	*Died*	*Number inoculated*	*No reaction*	*Reacted*	*Died*	*Number inoculated*	*No reaction*	*Reacted*	*Died*
Fibroma ..	1	..				8	0	8	8	4	0	4	4
	2					22	11	11	0	24	0	24	6
	3	..				28	16	12	0	14	1	13	0
	4	3	0	3	0	31	21	10	0	36	24	12	0
	5	..				9	4	5	0	15	12	3	0
	6	..								3	1	2	0
	7	3	0	3	0					8	5	3	0
	8	..				..				10	3	7	0
Myxoma ..	1	5	0	5	5								
	2	5	0	5	5								
	3	5	0	5	5	18	0	18	18	5	0	5	5
	4	8	0	8	8	5	0	5	3	5	0	5	5
	6 – 8	5	0	5	3	5	0	5	5	0	5	5	5
	9 –12	20	0	20	20	20	0	20	20	20	0	20	20

advertently provided as experimental subjects, frequently handled and unsuitably housed. These observations were restricted to a very small number of does.

TABLE 5

IMMUNITY TESTS ON RABBITS, THE PROGENY OF VACCINATED AND NORMAL DOES

Vaccinated when 2 – 7 weeks of age.					
Age at vaccination	*Doe*	*Number challenged*	*Result of challenge*		
			I.	*R.*	*D.*
2	Vaccinated	5	1	4	0
3	,,	5	1	0	4
4	,,	5	0	0	5
4	Normal	1	0	1	0
5	Vaccinated	13	8	4	1
6	,,	19	12	5	2
7	,,	34	29	4	1
7	Normal	13	8	4	1

I=Immune. R=Recovered. D=Died.

Comparison of the Protection against Myxomatosis Afforded by Two Substrains of the Boerlage Strain of Fibroma Virus and Using Two Routes of Inoculation

It was desirable to make a comparison between the two substrains, "P" and "F," and the different routes of inoculation. Accordingly, a vaccine-concentrate was prepared from substrain "F" and suitable dilutions were used on three occasions. On two of these, batches of strain "P" were also prepared by dilution and used on some of the rabbits. The dose was 0·5 cc., given subcutaneously to some, intradermally to others. The reactions to the vaccinating dose and the results of challenge 30 to 195 days later with virulent myxoma virus were noted.

The results, detailed in Table 6, show that 50 out of 70, or 71·4 per cent, of rabbits inoculated with the "F" substrain reacted to the fibroma virus, as compared with 45 out of 60, or 75 per cent, of those which received the "P" substrain. This difference is probably of no significance. On the other hand, 49 of the 60, or 81·7 per cent, of the rabbits inoculated intradermally reacted, as compared with 46 of the 70, or 65·7 per cent, of the rabbits inoculated subcutaneously, a difference which suggests that the intradermal route provokes more severe reactions.

Unfortunately, a number of the rabbits, kept for challenge from the 100th day onwards, died. However, results were available from

50 rabbits that had received the "F" substrain and from 38 that had received the "P" substrain. They suggest that the "F" substrain produced a better immunity since 14 per cent succumbed to challenge, as compared with 26 per cent vaccinated with the "P" substrain, and that the intradermal route gave a better protection (93 per cent) than the subcutaneous (78 per cent).

TABLE 6

SUMMARY OF THE RESULTS OF THE COMPARATIVE TESTS ON THE SUBSTRAINS OF SHOPE'S FIBROMA VIRUS

Fibroma strain	*Numbers of rabbits inoculated*	*Date*	*Route*	*No. reacted*	*Non-specific mortality*	*No. challenged*	*Period between vaccination and challenge (days)*	*Result*		
								I.	*R.*	*D.*
Australian 'F'	10	19.5.54	i/d	9	1	7	101	0	6	1
						2	195	1	1	0
	20	17.6.54	s/c	15	8	6	30	5	1	0
						4	104	3	1	0
						2	166	1	1	0
	10	17.6.54	i/d	10	4	6	104	5	1	0
	15	29.12.54	s/c	7	5	10	169	1	4	5
	15	29.12.54	i/d	9	2	13	169	7	5	1
	70			50				23	20	7
French 'P'	20	17.6.54	s/c	20	8	6	30	5	1	0
						6	104	4	1	0
	10	17.6.54	i/d	10	8	2	104	1	1	0
	15	29.12.54	s/c	4	3	12	169	1	5	6
	15	29.12.54	i/d	11	3	12	169	6	3	3
	60			45				17	11	9

	'F'	'P'
Total number of rabbits inoculated with each strain ..	70	60
Non specific deaths	20	22
Number challenged in each group	50	38
Total immune	23 (46%)	17 (45%)
Total recovered	20 (40%)	11 (29%)
Total mortality	7 (14%)	10 (26%)
Intradermal inoculation: Total	28	14
Immune	13(46·5%)	7(50%)
Recovered	13(46·5%)	4(28·6%)
Died	2(7%)	3(21·4%)
Subcutaneous inoculation: Total	22	24
Immune	10(45%)	10(42·6%)
Recovered	7(33%)	7(28·7%)
Died	5(22%)	7(28·7%)

DISCUSSION

Fibroma virus, in the form of a concentrate vaccine, was found to be viable, when kept either frozen at —40°C. or freeze-dried, for over a year. Although, by freeze-drying, the keeping properties of the vaccine can be greatly extended, the increased cost of producing such a preparation would probably make its price uneconomic. The fresh vaccine has, if properly handled, a life of fourteen days, and this should be ample for use in a country with good communications.

It is clear from the work of Andrewes (1936) that the virus of Shope's fibroma is liable to mutate into a strain giving an atypical reaction; thus, it was necessary to passage the original "P" strain as little as possible. It may be stated that in all the rabbits inoculated at Weybridge during two and a half years, no lesions have been detected suggesting a mutation of the virus in the direction of the inflammatory type of the original fibroma virus reported by Andrewes (1936).

It is generally recognised that some rabbits which have been inoculated with Shope's fibroma virus are completely resistant to inoculation of myxoma virus, others react to the inoculation of myxoma virus, often quite severely, but eventually recover, while a third group behave like fully-susceptible rabbits dying within the normal period after challenge. Most of those that are protected probably remain resistant for twelve months (Fenner and Woodroofe, 1954). Revaccination every six months is recommended if myxomatosis continues as a threat to tame rabbits in any area.

Comparison between the protection against myxomatosis afforded by the "P" and "F" substrains of Shope's fibroma, indicated the superiority of the latter. The results recorded in Table 6 suggest that the "F" substrain produced a better immunity since 14 per cent succumbed to challenge as compared with 26 per cent vaccinated with the "P" substrain. Fenner and Woodroofe (1954) reported that, using 1,000 ID/50 of their "F" substrain of the Boerlage strain given intradermally, they had immunized seventeen rabbits; representative animals drawn for challenge 8 to 52 weeks afterwards invariably survived. Seven had no local lesion and most of the others developed no secondary manifestations. These results were considerably better than those reported in the present paper. Intradermal inoculation gave 14·2 per cent more protection than subcutaneous.

In some virus diseases the offspring of an immune dam receive antibodies in the colostrum or through the placenta. While such antibodies are circulating in their blood, the young animals will not react to the inoculation of a vaccinating dose of virus contained in a living virus vaccine. Results recorded in Table 4 indicated that no protection is afforded to the young of myxoma-immune does against inoculation of either fibroma or myxoma viruses. During the first

week of life it is unsafe to vaccinate any young rabbits. During the second week, although the young, born to a vaccinated doe, can be inoculated without risk, the offspring of a susceptible doe may still die following treatment. Hyde and Gardner (1939) have reported deaths in rabbits inoculated before they were more than three days old. In very young rabbits which die, the fibroma virus generalizes and a clinical picture is produced similar to that recorded as developing very rarely in older, vaccinated domesticated rabbits, by Jacotot, Vallée and Virat (1954a).

CONCLUSIONS

The paper describes in detail experiments made to answer practical questions that arise in regard to vaccination of tame rabbits against myxomatosis with Shope's fibroma virus.

ACKNOWLEDGMENT

We are grateful to Dr. H. Jacotot of the Institut Pasteur, Paris, and Professor F. Fenner, Australian National University, for the provision of strains of virus, and to the former for much useful information on the routine preparation of vaccine.

REFERENCES

Andrewes, C. H. (1936). *J. exp. Med.*, **63**, 157.
Berry, G. P., and Litchty, J. A. (1936). *J. Bact.*, **31**, 49.
Fenner, F., and Woodroofe, Gwendolyn, M. (1954). *Aust. J. exp. Biol.* **32**, 653.
Hurst, E. W. (1938). *Ibid.*, **16**, 265.
Hyde, R. R. (1939). *Amer. J. Hyg.*, **30**, 47.
Hyde, R. R., and Gardner, R. E. (1939). *Ibid.*, 57.
Jacotot, H., and Vallée, A. (1953). *Ann. Inst. Past. Paris.*, **85**, 133.
Jacotot, H., Vallée, A., and Virat, B. (1954a). *Bull. Acad. vét. Fr.*, **27**, 105; (1954b). *Ibid.*, 465.
Martin, C. J. (1936). *Bull. Coun. sci. indust. Res. Aust.* No. 96.
Ritchie, J. N., Hudson, J. R., and Thompson, H. V. (1954). *Vet. Rec.*, **66**, 796.
Shope, R. E. (1936). *J. exp. Med.*, **63**, 33; *Ibid.*, 43; (1938). *Proc. Soc. exp. Biol. N.Y.*, **38**, 86.

[*Received for publication, February 15th,* 1956]

EFFECTS OF FEEDING FURAZOLIDONE TO FOWLS.

I. Effect on Body Weight and Sexual Development in Cockerels

By

Doreen M. Cooper

Houghton Poultry Research Station, Houghton, Huntingdon

and

G. Skulski

Canine Research Station, The Animal Health Trust, Newmarket

INTRODUCTION

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) has been shown to be effective in the control of experimental fowl typhoid (Smith, 1954a, b) and experimental *Salmonella pullorum* infections (Smith, 1954; Gordon and Tucker, 1955) in chickens. It also appears to be of considerable value as a preventative of histomoniasis in turkeys (Horton-Smith and Long, 1956). Continuous feeding of the drug was reported by Jacobs, Elam, Anderson, Gee, Fowler and Couch (1953) to have no stimulatory effect on body weight when fed to chicks from day-old to ten weeks of age held in a clean environment. On the other hand, Harwood and Stunz (1954a) noted a growth stimulation similar to the "antibiotic effect" when the drug was added to all-mash rations at levels of 0·0055 to 0·02 per cent and this was most pronounced under conditions of poor sanitation (Harwood and Stunz, 1954b). When the drug was added to the ration at the rate of 0·033 per cent, however, there was a retardation in growth which became significant after four weeks continuous medication.

The LD_{50} for furazolidone has been calculated to be between 400 and 500 mg/kg. for both chickens and turkeys (Harwood and Stunz, 1954a). Smith (1955b) observed nervous symptoms in chicks which had been fed the drug at 0·2 per cent w/w during a period of 1 to 13 days. Similar toxic effects have been observed in mice (Hobson, 1956).

Little research has been undertaken to determine whether side effects occur following the continuous feeding of furazolidone at low levels and what are the effects of high level dosage over short intervals. An abstract of a paper by Moore, Chamberlin and Carter (1954) reported no adverse effect on egg production, fertility and hatchability in turkeys, but the dosage was not given. In a preliminary communication, Cooper and Skulski (1955) reported that the continuous feeding of furazolidone to Single Comb White Leghorn cockerels from day-old to twelve weeks of age resulted in a significant reduction in the weight of testes taken from birds fed the drug at the 0·02 per cent level. Although no significant reductions in testes weights occurred in birds fed the drug at the lower levels,

histological examination revealed a retardation of spermatogenesis. Since there is a great probability, in spite of warnings, that the drug will be used in breeding flocks, it was felt that an investigation into the effects of this drug on sexual development should be undertaken. This paper reports the results of a number of experiments in which furazolidone was fed at levels used in prevention, treatment and up to twice the concentration used in the treatment of established disease.

MATERIALS AND METHODS

Mature and immature Single Comb White Leghorn chickens hatched at this station were used as the test animals in all experiments and their numbers and ages are detailed under each experiment. The birds were housed in experimental units which permitted them to be kept in the same environment throughout the whole of the experimental period. They were fed a chick starter ration (Horton-Smith and Long, 1956) from day-old to 14 weeks and the station breeder ration from 14 weeks to maturity. Furazolidone was added to the ration according to the procedure described by Horton-Smith and Long (1956). The levels of drug varied from 0·005 to 0·02 per cent w/w.

Birds were sacrificed by dislocation of the vertebrae. The testes were removed, weighed and placed in 10 per cent formol-saline for 24 to 48 hours. Testicular tissue from at least three of the six to eight birds sacrificed from each group (representing those with the largest, smallest and average-sized testes) was embedded in paraffin. Sectioning was singly and serially.

Sections were stained routinely with haematoxylin-eosin. Other methods included Picro-Mallory for hyaline changes; Congo-red method of Bernhold for amyloid degeneration; Southgate's muci-carmine method for mucus; Bauer's method for glycogen; von Kossa's method for calcium and Putt's carbol-fuchsin method for spermatozoa.

RESULTS

General

The Effect on Body Weight

Day-old chicks, randomized into five groups of 30 and brooded by means of infra-red lamps of the dull emitter type, were fed furazolidone at 0, 0.005, 0.01, 0.015 and 0.02 per cent w/w for a twelve week period. The chicks were weighed at day-old and at weekly intervals. Sexing was carried out at the conclusion of the experiment. There were no significant differences in body weight between treatments (Table 1). Since the birds were housed under ordinary experimental conditions, i.e. in houses which had been used for considerable periods but which were thoroughly cleaned and rested before use, these results indicate that the drug does not exert any stimulatory effect on body weight, in agreement with the results obtained by Jacobs, *et. al.* (1953).

TABLE 1

THE EFFECT OF CONTINUOUS FEEDING ON BODY WEIGHT

Body weight Grams	*Dose of furazolidone*				
	Controls	*+0·005%*	*+0·01%*	*+0·015%*	*+0·02%*
Day-old	(Corrected weight) 35				
12 weeks	1143	1189	1211	1212	1149

Difference in treatments —Not significant.
Treatment x sex interaction—Not significant.
Difference between sexes —Highly significant (P=<0·001).

The Effect of Continuous Feeding from Day-old to Maturity on Tissue Weight and Development (Group 2)

Day-old cockerel chicks were randomized into five groups of 25 chicks each. Furazolidone was added to the ration at 0, 0·005, 0·01, 0·015 and 0·02 per cent w/w. After 12, 18 and 24 weeks, one-third of each group was weighed and the birds sacrificed. The comb was removed close to the head. Comb and wattles were weighed separately. The testes were removed, weighed and placed in fixative. The results of this experiment (Table 2) show that although there were no significant differences between the controls and treated groups with regard to comb and wattle weights, a significant reduction in testes weight did occur in the group fed the drug at the 0·02 per cent level following feeding for twelve weeks (P=<0·01). The effect of the drug on reducing testes weight was also pronounced with groups fed the drug at 0·02 and 0·015 for 18 weeks.

The Effect of Feeding Continuously from Six to Eighteen Weeks of Age followed by Discontinuance (Group 3)

This experiment was designed to see if age at commencement of drug treatment produced a difference in effect. Day-old cockerels were randomized into five groups of 25 chicks each and treated as in the previous experiment except that drug treatment did not commence until the birds were six weeks old. At the end of treatment (12 weeks) and at six and twelve weeks following discontinuance of the drug, one-third of the group were sacrificed and the same measurements carried out to see whether the changes found in birds immediately after the conclusion of treatment were permanent or whether a return to normal spermatogenesis occurred when it was discontinued. The period of twelve weeks was chosen arbitrarily in view of the report by Pino and Hudson (1953) that normal spermatogenesis was evident ten weeks following discontinuance of Enheptin. The results of this experiment are shown in Table 3. At the end of the drug treatment no statistically significant reduction in testes weight was observed in the treated

TABLE 2

THE EFFECT OF CONTINUOUS FEEDING ON BODY, TESTES, COMB AND WATTLE WEIGHTS

	At 12 weeks				*At 18 weeks*				*At 24 weeks*			
	Body wt. gm.	*Testes wt. mg./kg.*	*Comb wt. gm.*	*Wattle wt. gm.*	*Body wt.*	*Testes wt. mg./kg.*	*Comb wt.*	*Wattle wt.*	*Body wt.*	*Testes wt. mg./kg.*	*Comb wt.*	*Wattle wt.*
Controls	1323·9	6468	9·10±0·73	3·54±0·25	1774·7	11825	20·28±0·97	7·36±0·41	2159·6	7923	28·04±2·30	9·74±0·50
+0·005%	1327·9	6119	8·43±1·48	3·35±0·75	1885·6	9047	22·07±1·34	7·18±0·70	2034·8	9342	26·33±1·80	10·33±0·53
+0·01%	1406·9	6668	10·21±1·10	3·90±·004	1867·5	10162	21·98±1·60	7·34±0·57	2088·0	7626	25·56±1·86	10·65±0·50
+0·015%	1401·4	4231	9·69±1·38	3·52±0·03	1849·1	*7324	20·57±2·00	6·38±0·51	2069·4	7279	26·20±2·35	11·25±1·11
+0·02%	1431·6	**2614	7·19±0·69	3·42±0·41	1814·2	*7749	20·19±1·28	7·89±0·49	2160·4	7536	21·76±1·88	10·61±0·59

**P=<0·01. *P=<0·02>0·01.

groups, nor were there significant differences in comb and wattle weights.

The Effect of Feeding High Levels for Short Periods to Mature Cockerels (Group 4)

Cockerels of 24 weeks of age were randomized into three groups of 15 birds each and housed in experimental units fitted with slatted floors. Before the experiment commenced the birds were weighed and handled to obtain semen in order to ensure that sexual maturity had been reached. Furazolidone was added to the breeders ration at 0·04 and 0·08 per cent w/w and the medicated mash was fed for 21 days. Eight birds from each group were sacrificed at the end of the treatment, and the remainder four weeks later. Measurements were made as before. In this experiment testes from all birds were sectioned.

The effect of feeding furazolidone at the 0·08 per cent level was most pronounced, the birds showing a marked decrease in comb and wattle size and a highly significant decrease in testes weight (Table 4). Although it was not possible under our experimental conditions to estimate accurately the food consumption, there was a definite decrease (25 to 30 per cent) in food consumption by the birds receiving the drug at this level. Following discontinuance of the drug, food consumption during a four week period was essentially the same in all groups. A significant decrease in size of secondary sex characteristics was not apparent in the birds fed the drug at the 0·04 per cent level, but a significant decrease in testes weight did occur ($P = <0{\cdot}05 > 0{\cdot}02$). During the four week period following discontinuance of the drug it became apparent within a week that the secondary sex characteristics were returning to normal in the group fed the drug at the 0·08 per cent level. From the results in Table 4b it would seem that a longer period than four weeks is necessary for comb and wattles to return to normal size, although testes weights by then were comparable to those in the controls. Food consumption returned to normal within a week after withdrawal of medicated feed.

The Effect of Feeding High Levels to Immature Cockerels for 14 days (Group 5)

Five-week old cockerels were randomized into four groups of 13 birds each. Furazolidone was added to chick mash at levels of 0, 0·04, 0·06 and 0·08 per cent w/w. The birds were weighed at the beginning of the experiment and measurements made of the comb area (length x height cm.). A record was kept of food consumption. At the end of the treatment the birds were weighed, comb measurements recorded and eight birds sacrificed. The remaining birds were again weighed and measured before being killed four weeks later. In this experiment testes from all birds were sectioned.

The results of this experiment are shown in Table 5. The effect

TABLE 3

THE EFFECT OF CONTINUOUS FEEDING ON BODY, TESTES, COMB AND WATTLE WEIGHTS

Period 6 to 18 weeks of age.

Treatment	*At end of drug treatment (18 weeks of age)*				*Six weeks following discontinuance of treatment (24 weeks of age)*				*Twelve weeks following discontinuance of treatment (30 weeks of age)*			
	Body wt. gm.	*Testes wt. mg./kg.*	*Comb wt. gm.*	*Wattle wt. gm.*	*Body wt. gm.*	*Testes wt. mg./kg.*	*Comb wt. gm.*	*Wattle wt. gm.*	*Body wt. gm.*	*Testes wt. mg./kg.*	*Comb wt. gm.*	*Wattle wt. gm.*
Controls	2068·4	7828	20·99±1·79	7·92±0·86	2145·4	8485	25·88±2·00	10·16±1·37	2416·0	5524	26·21±2·88	9·12±1·34
+0·005%	2055·4	7022	16·81±1·57	6·73±0·90	2135·5	8412	26·41±1·93	10·58±1·43	2438·0	5031	26·79±1·88	11·86±2·14
+0·01%	2051·5	6019	20·20±2·29	7·10±0·79	2146·7	7489	24·31±1·75	10·22±0·54	2416·0	6454	33·35±1·35	12·73±0·49
+0·015%	1948·4	7499	20·11±1·82	7·99±0·66	2300·1	8130	26·51±2·32	11·79±0·87	2427·2	*2994	21·20±1·44	8·28±1·26
+0·02%	2009·6	6309	16·89±2·01	6·85±0·84	2287·9	8109	25·40±2·36	10·63±1·25	2469·0	6116	29·55±1·96	11·37±0·84

*P=<0·05>0·02.

TABLE 4

THE EFFECT OF FEEDING HIGH LEVELS TO MATURE COCKERELS

Period of 21 days.

(a) At end of 21 day treatment.

Treatment	*Body wt. gm.*		*Testes wt.*		*Comb wt. gm.*	*Wattle wt. gm.*
	Initial	*at 21 days*	*Gm.*	*Mg./kg.*		
Controls	2243·1	2318·6	16·05±1·55	7256	29·76±2·42	8·51±0·73
+0·04% NF 180 ..	2386·5	2454·8	11·54±2·14	4595*	29·39±2·96	8·92±1·14
+0·08% NF 180 ..	2227·2	2061·3	2·93±0·71**	1444**	15·18±1·75**	4·70±0·42**

** P=<0·001. * P=<0·05>0·02.

(b) Four weeks following discontinuance of drug.

Treatment	*Body wt. gm.*		*Testes wt.*		*Comb wt. gm.*	*Wattle wt. gm.*
	At 21 days	*4 wks. following discont. drug*	*Gm.*	*Mg./kg.*		
Controls	2318·6	2623·0	14·91±1·94	5679	37·29±3·02	11·36±1·54
+0·04% NF 180 ..	2454·8	2612·0	18·05±1·61	6897	32·74±4·26	10·79±0·88
+0·08% NF 180 ..	2061·3	2529·0	14·14±2·12	5546	**26·16±1·67	*7·54±0·59

** P=<0·01>0·001. * P=<0.05>0·02.

of the drug at the 0·06 and 0·08 per cent levels was most marked in all measurements including body weight. A reduction in body weight in chicks fed the 0·04 per cent level was also highly significant, and the effect on comb weights was significant between the 5 and 2 per cent endpoints. Food consumption was somewhat reduced in groups 0·04 and 0·06 per cent, and a 30 per cent reduction in food consumption in the 0·08 per cent group was recorded. During the four week period following discontinuance of the drug there were no real differences in food consumption. The data at the end of this period show that body weight had not returned to that of the controls, but testes weight, comb and wattle weights showed no significant differences.

Histology

Group 2

Changes found in groups fed the drug at 0·005, 0·01 and 0·015 per cent were not of great significance. Some disorder was noted in cells in the seminiferous tubules. Interstitial tissue was increased and in some cases hyperaemia was noted. The numbers of spermatocytes, spermatids and spermatozoa were normal. In the group fed 0·02 per cent striking changes were observed in the form of an increase in interstitial tissue with hyperaemia as well as disorder in the arrangement of cells. Some tubules showed a lack of spermatogonia, primary and secondary spermatocytes and spermatids and contained a scanty number of spermatozoa. Others showed complete atrophy or complete disorder in the arrangement of cells (Fig. 2).

Group 3

Sections were studied from birds fed the drug from 6 to 18 weeks of age and sacrificed (i) immediately after the end of treatment, (ii) six weeks thereafter, (iii) 12 weeks thereafter.

Although changes occurred at all levels they were not of great significance but some disorder in the arrangement of cells was present in all sections. At 0·01, 0·015 and 0·02 per cent there was an increase in amount of intersititial tissue and numbers of R.B.C.'s in the lumen of the seminiferous tubules. In some tubules there was a lack of secondary spermatocytes and/or spermatids. The numbers of spermatozoa were normal except in birds fed 0·02 per cent in which they were scanty. Sertoli cells were diminished in number in sections from the 0·015 and 0·02 per cent levels.

In sections from birds fed the drug at 0·005 and 0·015 per cent levels, in addition to the changes found above, slight perivascular hyaline changes were seen.

Although all types of cells were recognized in the seminiferous tubules their arrangement in the majority of tubules was affected. In some the lumen was completely packed with primary and secondary spermatocytes and spermatids. The numbers of sperma-

TABLE 5

THE EFFECT OF FEEDING HIGH LEVELS TO IMMATURE COCKERELS

14 days.

(a) At end of 14 day treatment.

Treatment	*Body wt. gm.*	*Gain over initial wt.*	*Testes wt.*		*Comb wt. gm.*	*Comb area cm²*	*Difference in comb area cm²*	*Wattle wt.*
			gm.	*mg./kg.*				
Controls ..	599·7	227·7	0·355	605	3·28±0·44	13·92	+7·38	0·916±·010
+0·04% ..	525·7	*162·3	0·201	370	†2·27±0·29	10·06	+4·80	0·623±0·10
+0·06% ..	416·9	**50·4	0·083	‡208	**1·09±0·06	5·92	+1·03	**0·296±0·21
+0·08% ..	472·3	**94·8	0·097	‡204	**1·01±0·04	6·71	+2·41	**0·370±0·03

† P=<0·05>0·02. ‡ P=<0·02>0·01. * P=<0·01>0·001. ** P=<0·001.

(b) Four weeks following discontinuance of drug.

Treatment	*Body wt. gm.*	*Gain over initial wt.*	*Testes wt.*		*Comb wt. gm.*	*Comb area cm²*	*Difference in comb area cm²*	*Wattle wt.*
			gm.	*mg./kg.*				
Controls ..	1215·4	836·8	0·61±0·16	487	6·94±0·95	26·60	+20·07	2·15±0·35
±0·04% ..	1114·0	736·4	1·09±0·43	985	5·18±0·48	21·18	+16·02	1·42±0·40
+0·06% ..	1022·2	*665·0	0·324±0·02	319	3·69±0·30	17·61	+13·42	1·42±0·07
+0·09% ..	1085·2	†712·8	0·476±0·10	430	4·67±0·86	18·86	+13·80	1·51±0·13

† P=<0·05>0·02. * P=<0·01.

tozoa were scanty. The tubules were irregular in size and the interstitial tissue was increased and showed hyperaemia. Slight hyaline deposits were noted around blood vessels. In the 0·015 and 0·02 per cent groups changes similar to those in birds fed the 0·005 and 0·01 per cent levels were observed. The most striking of these occurred in the 0·015 per cent group in which there was a lack of primary and secondary spermatocytes and spermatids. Some tubules showed a normal or scanty number of spermatozoa. Other tubules were atrophied with few or no cells in the lumen (Fig. 7 a, b). Sertoli cells were diminished in number especially in sections taken from birds which had received furazolidone at concentrations of 0·015 and 0·02 per cent.

Group 4

Tissues from birds were collected immediately following discontinuance of the drug (group 1) and at 4 weeks thereafter (group 2).

In the first, disorderly arrangement of cells in the seminiferous tubules was observed in sections from birds fed the drug at 0·04 and 0·08 per cent levels. There was a great amount of fibrous tissue causing the disappearance of the seminiferous tubules. The majority of tubules showed a lack of all cells, but a few contained spermatozoa. They were either hypertrophied or atrophied and in the latter case showed debris of degenerating spermatozoa (Fig. 3, 4).

In the second, the changes seen were a disorderly arrangement of cells, hyperaemia, hypertrophy and atrophy of seminiferous tubules. There was a lack of primary and secondary spermatocytes, spermatids and spermatozoa. A slight hyaline deposit was also noticed. In tubules which did not show atrophy the arrangement of cells was normal (Fig. 6).

Group 5

Sections taken immediately following discontinuance of treatment from birds fed the drug at 0·04 per cent level showed cellular infiltration of interstitial tissue. At the 0·06 and 0·08 per cent levels, there was, in addition, a large amount of fibrous tissue with resulting disappearance of the seminiferous tubules. At these levels the lumen of some of them was filled with an eosinophilic mass of unknown origin. The methods of staining used failed to demonstrate the nature of this substance. The atrophied tubules were devoid of all cells, suggesting a complete lack of spermatogenesis, (Fig. 5).

DISCUSSION

Although the primary requisite in recommending a drug in the treatment of disease is its efficacy in eliminating the causative organism, it is nonetheless important to know whether it has any adverse effect on other body organs, and if so, whether such changes

are of a temporary or permanent nature. Furthermore, it is necessary to know whether such changes are limited to either sex.

The experiments reported in this paper comprised birds of three age groups (i) chicks from day-old to maturity (ii) young birds 5 to 6 weeks of age and (iii) mature males. Drug treatment included the continuous feeding of the drug at low levels as would be recommended in preventive treatment and the feeding of high levels of the drug for a short time as recommended in curative treatment. In choosing birds of 5 to 6 weeks of age it was hoped to determine whether the drug fed at a time when a rapid development of gonadal tissue was imminent had a more pronounced effect on this tissue in them than in those of other age groups. S.C.W.L. males were chosen because this breed is more sexually precocious than the heavy breeds, semen production commencing at 13 weeks of age (Lorenz and Lerner, 1946), and it has been shown that the comb and wattles are a more sensitive indicator of androgen activity (Dorfman and Dorfman, 1948).

Of particular interest is the effect of low levels of furazolidone on testicular development without a significant effect on comb and wattle growth. It has been shown that with certain doses of X-rays the testes may be reduced in size and tubular dysfunction occur, but the secretion of androgen is apparently normal (Sturkie, Pino, Weatherwax, Donnelly and Dorrance, 1949). It is therefore necessary that histological examination of such tissues be carried out before it can be stated that the drug has no effect on the gonads.

When large doses of furazolidone were fed, both testes weight and secondary sexual characteristics were suppressed. In these cases the increase in the amount of interstitial tissue was noted in certain areas of the testicular tissue only. Since the greatest volume of the testes is occupied by the seminiferous tubules, which, being atrophied, account for the reduction in testes size, the hypertrophy of the intersititial tissue is insufficient to compensate for this factor as far as macroscopical examination is concerned. It is not known whether the hormone secretion or numbers of spermatozoa remain normal since our experiments were designed only to study the macro- and microscopic changes in the testes produced by feeding furazolidone. It seems probable however, that the secretion of hormones was reduced because histological examination revealed a diminished number of Sertoli cells in the testes and in some atrophied areas a lack of Leydig cells in the interstitial tissue.

Although histological study shows that this drug has a marked effect on the testes, it cannot be stated with certainty that the changes are of a permanent nature as recovery, if it occurs, may take considerable time. Whether these changes are sufficient to cause a marked reduction in fertility has not been determined but it seems possible that some reduction may occur. Further research into this aspect will be undertaken.

CONCLUSIONS

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) was fed continuously to S.C.W.L. male chicks from day-old to maturity and to males from 6 to 18 weeks of age at levels of 0·005 to 0·02 per cent. The drug was fed at high levels (0·04 to 0·08 per cent) to 5 weeks old males for 14 days and to mature males for 21 days. Measurements of the effect of the drug on body weight, comb and wattle weight, and testicular development were recorded. Histological studies of the effect of the drug on the testes were also undertaken.

It has been shown that the continuous feeding of the drug may effect histological changes in the testes even though there are no significant differences in testes weight. Some changes were still apparent even after the feeding of the drug at low levels had been discontinued for twelve weeks. When high levels of the drug were fed for short periods to both immature and mature males, a marked effect, both macro- and microscopically was noted. Although such effects were still apparent four weeks following discontinuance of the drug, some tubules did show a normal arrangement of cells.

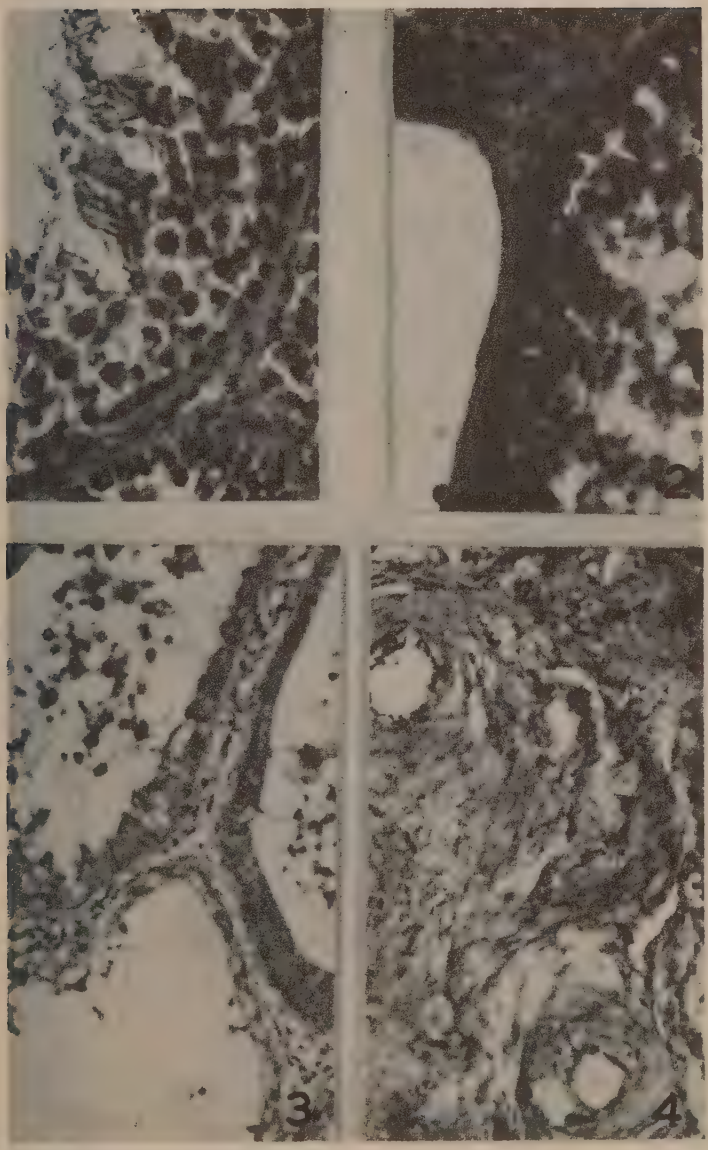
It has not yet been determined whether the birds fully recover from the effects produced by feeding, nor has it been possible to estimate the length of time required to return to normal spermatogenesis. It does appear, however, to require more than twelve weeks following discontinuance of the drug.

ACKNOWLEDGMENTS

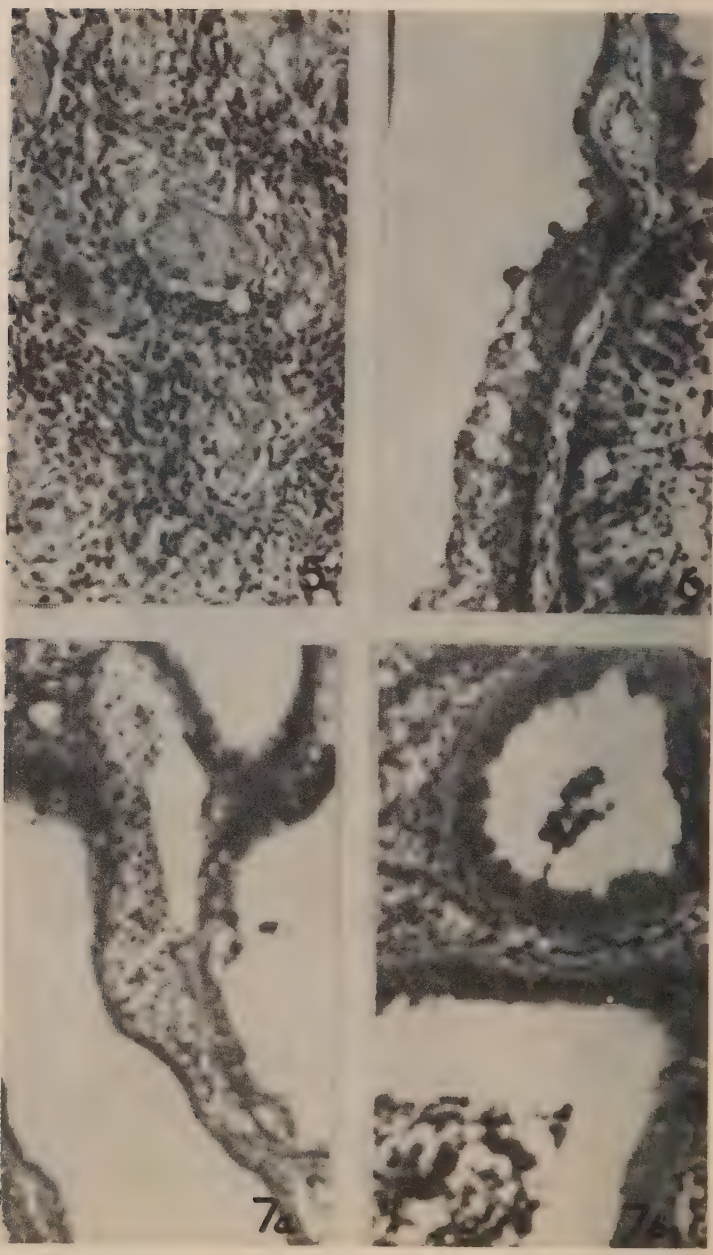
We should like to express our appreciation ot Mr. W. G. Orbell for the microphotographs, to Mr. J. G. Rowell, Agricultural Research Council Unit of Reproduction, for the statistical analysis of Experiment 1, and to Mr. C. Hart and Miss P. Reynolds for technical assistance. Our thanks are due to Mr. S. F. J. Hodgman, M.R.C.V.S. for permitting one of us (G.S.) to work on this project, and to Dr. Horton-Smith for his helpful criticism in the presentation of the paper. Furazolidone was provided through the courtesy of Messrs. A. J. White Ltd., Coldharbour Lane, London.

REFERENCES

Cooper, D. M., and Skulski, G. (1955). *Vet. Rec.*, **67**, 541.
Dorfman, R. I., and Dorfman, A. S. (1948). *Endocrinology*, **42**, 7.
Gordon, R. F., and Tucker, J. (1955). *Vet. Rec.*, **67**, 116.
Harwood, P. D., and Stunz, D. I. (1954a). *Proc. Xth World's Poultry Congress, Edinburgh*, 222.
Harwood, P. D., and Stunz, D. I. (1954b). *J. Parasitol.*, **40**, 24.
Hobson, D. (1956). *Brit. J. exp. Path.*, **37**, 20.
Horton-Smith, C., and Long, P. L. (1956). *J. comp. Path.*, **66**, 22.
Jacobs, R. L., Elam, J. F., Anderson, G, W., Gee, L. L., Fowler, J., and Couch, J. R. (1953). *J. Nutrit.*, **51**, 507.
Lorenz, F. W., and Lerner, I. M. (1946). *Poult. Sci.*, **25**, 188.
Moore, E. N., Chamberlin, J. D., and Carter, R. D. (1954). *Ibid.*, **33**, 1072 (abst.)



To face page 310


5
6
7a
7b

Pino, J. A., and Hudson, C. B. (1953). *Ibid.*, **32**, 650.
Smith, H. Williams. (1954a). *Vet. Rec.*, **66**, 215; (1954b). *Ibid.*, 493; (1955a). *J. comp. Path.*, **65**, 55; (1955b). *Personal communication.*
Sturkie, P. D., Pino, J. A., Weatherwax, J. L., Donnelly, A. J., and Dorrance, G. M. (1949). *Radiology*, **52**, 112.

[*Received for publication, February 21st,* 1956]

KEY TO PHOTOGRAPHS

Fig. 1. Normal testes in the mature male, showing arrangement of primary and secondary spermatocytes, spermatids and spermatozoa. × 200.

Fig. 2. Testes from 24 week-old cock continuously fed with 0·02% furazolidone. One seminiferous tubule (left) shows complete atrophy and disappearance of cells; that on the right shows a disorderly arrangement of cells. × 200.

Fig. 3. Testes from mature bird fed 0·06% furazolidone for 21 days. Atrophy of tubules, with cells absent or few in number and lying in disorder. × 100.

Fig. 4. Testes from a mature bird fed 0·08% furazolidone for 21 days. Complete disappearance of tubules, greatly increased amount of interstitial tissue and large amount of fibrous tissue. At top left and bottom right, two seminiferous tubules reduced in size and with a complete lack of cells. × 100.

Fig. 5. Testes from a 7 week old cock fed 0·08% furazolidone for 14 days. Atrophy of seminiferous tubules, great amount of fibrous tissue and increased amount of highly cellular interstitial tissue. An eosinophilic material may be seen in the lumen of the tubules. × 100.

Fig. 6. Testes 4 weeks after discontinuance of the drug. Mature cock fed 0·08% furazolidone for 21 days. Atrophy of seminiferous tubules and disorder in the arrangement of cells. × 100.

Fig. 7. (a, b). Testes from a bird 12 weeks after the discontinuance of the drug, fed at 0·015% from 6 to 18 weeks of age.
7 a. Complete atrophy of the seminiferous tubules. × 100.
7 b. A few cells left in the lumen of the seminiferous tubules. × 100.

All sections stained with haematoxylin-eosin.

EFFECTS OF FEEDING FURAZOLIDONE TO FOWLS

II. Effect on Egg Production, Fertility and Hatchability of Low Level Feeding to Pullets

By

Doreen M. Cooper

Houghton Poultry Research Station, Houghton, Huntingdon

INTRODUCTION

The effect of feeding furazolidone (N-(5-nitro-2-furfurylidene)-3 amino-2-oxazolidone) on the gonad development in S.C.W.L.* cockerels has been reported by Cooper and Skulski (1955, 1956). Of equal interest is the effect of this drug on the female, especially in relation to egg production, fertility and hatchability. The experiment reported herein deals with the subsequent effect of low level drug feeding on these factors in S.C.W.L. pullets.

MATERIALS AND METHODS

Pullets hatched in March 1955 were randomised into four groups of 24 birds each when ten weeks of age. Each group was housed in an intensive breeding unit consisting of a small house with trapnests and having a slatted floored sunporch. Lighting was by means of a 60W. frosted lamp bulb, the birds receiving 14 hours light per day from 16 weeks of age until the end of the experiment.

Furazolidone was prepared in a 10 per cent. premix with plain flour and added to the breeders ration (Cooper and Skulski, 1956) at levels of 0·01, 0·015 and 0·02 per cent. The medicated ration was fed from 14 to 26 weeks of age. After 26 weeks of age the birds were fed a normal breeders ration. Food consumption was recorded.

The birds were weighed at 14 and 26 weeks of age. Egg production was recorded from the date of first egg until the termination of the experiment on December 26th, 1955. Fertility and hatchability were recorded for a period of 13 weeks. A record of mortality was kept.

The pullets were inseminated once weekly with 0·1 ml. of fresh, undiluted semen obtained from R.I.R.† males fed a normal breeders ration. Any effect by the drug would therefore be confined to the female. The hens were trapnested and the eggs set once weekly in a forced-draught incubator of the pre-heated air type. The eggs were candled on the 18th day and all eggs appearing 'clear' or showing evidence of dead germs were opened and examined macroscopically under a bright light for evidence of embryonic development.

* S.C.W.L. = Single Comb White Leghorn.

† R.I.R. = Rhode Island Red.

RESULTS

Body Weight

The average body weights of the birds at the beginning and end of the experiment are shown below.

Treatment	*Initial Wt. (gm.)*	*Final Wt. (gm.)*	*Gain (gm.)*
Controls	1168·2	1981·1	812·9
+0·01% furazolidone ..	1196·3	1937·1	740·8
+0·015% furazolidone ..	1225·6	2017·8	792·2
+0·02% furazolidone ..	1212·8	1930·5	717·7

Although there is some difference between the average gain between controls and the 0·02 per cent. treatment, statistical analysis by the "t" test shows this to be non-significant.

Feed Consumption

Feed consumption during the period 14 to 42 weeks is as follows:—

Treatment	*Period 14–26 wks. medicated mash*	*Period 27–42 wks. non-medicated mash*
Controls	20·1 lbs./bird	30·5 lbs./bird
+0·01% furazolidone ..	19·8 lbs./bird	29·5 lbs./bird
+0·015% furazolidone ..	20·8 lbs./bird	30·5 lbs./bird
+0·02% furazolidone ..	19·3 lbs./bird	29·0 lbs./bird

Egg Production

Egg production commenced while the birds were fed the medicated mash, the first egg from each group being laid within a period of three days. Egg production was calculated by four-week periods as follows:—

Treatment	*No. hens*	*Aug. 8*	*Sept. 5*	*Oct. 3*	*Oct. 31*	*Nov. 28*	*Dec. 26*	*Total eggs*	*Average hen-housed*
Controls ..	24	25	245	322	309	266	271	1438	59·9
+0·01% furazolidone	24	27	221	367	368	259	263	1505	62·7
+0·015% furazolidone	22	27	215	334	354	269	273	1472	66·9
+0·02% furazolidone	23	23	247	365	317	305	237	1494	64·9

TABLE 1

FERTILITY AND HATCHABILITY OF EGGS SET

	October						*November*						*December*						*Total*					
	No. set	*Inf.*	*Edg.* †	*Dis.* ‡	% *Fertile*	% *Hatch all eggs*	*No. set*	*Inf.*	*Edg.*	*Dis.*	% *Fertile*	% *Hatch all eggs*	*No. set*	*Inf.*	*Edg.*	*Dis.*	% *Fertile*	% *Hatch all eggs*	*No. set*	*Inf.*	*Edg.*	*Dis.*	% *Fertile*	% *Hatch all eggs*
Controls ..	371	120	16	11	67·6	60·3	304	34	6	11	88·8	83·2	268	61	14	25	77·2	62·7	943	215	36	47	78·2	68·4
+0·01% furazolidone	401	65	23	14	83·8	74·5	265	39	3	10	85·0	80·3	256	55	5	17	78·5	69·9	922	159	31	41	82·7	74·9
+0·005% furazolidone	427	64	19	27	85·0	74·2	273	38	8	13	86·1	78·3	268	48	9	21	82·1	70·9	968	150	36	61	83·4	74·4
+0·02% furazolidone	360	56	24	13	84·4	74·1	267	29	16	23	89·4	74·5	222	38	7	16	82·8	72·5	849	123	47	52	85·5	73·9

† Edg = Embryos dying by 96th hour of incubation. ‡ Dis = Chicks dead-in-shell.

Fertility and Hatchability

Fertility, early embryonic mortality to the 96th hour of incubation, and number of chicks dead-in-shell are shown in Table 1. Although the groups which had received the drug treatment appeared to give slightly better fertility, this was due to a somewhat lower fertility obtained during the first two weeks of insemination and this small difference is of no statistical significance.

Mortality

The causes of mortality were as follows:—

Treatment	*No.*	*Diagnosis*	
Controls	1	Nephritis	1
+0·01% furazolidone ..	5	Neural lymphomatosis	3
		Lymphoid leucosis	1
		Nephritis	1
0·015% furazolidone ..	4	Neural lymphomatosis	1
		Visceral lymphomatosis	1
		Septicaemia of unknown origin	1*
		Cannibalism	1
0·02% furazolidone ..	3	Lymphoid leucosis	1
		Chronic indigestion	1
		No diagnosis	1

*Bird died between 10 and 14 weeks of age. Not fed medicated mash.

DISCUSSION

Drugs which are to be used for preventative treatment should not only be effective in the prevention of the disease but should also be of such a nature that they do not produce any adverse side effects, even if they have to be used continuously over a long period of time.

The pullets in this experiment were fed the drug from 14 weeks until they were 26 weeks of age to determine whether low level feeding, as recommended in preventative treatment, would delay the onset of egg production and/or affect total egg production. The hens were inseminated with semen from untreated males to enable the observation to be made of the effect of the drug on the reproductive capacity of the female only and not be complicated by any effect the drug may have on the reproductive system of the male bird.

Egg production in the control group and in two of the treated groups commenced on the same day and in the third treated group, three days later. The birds were 19 weeks of age at commencement of lay, and this may be regarded as average age at onset of egg production. Although it may appear that the birds which had been fed the drug gave slightly better fertility, this is of no statistical

significance and is due to the rather low fertility in the controls following the first three inseminations.

From the results of this experiment it appears that the mode of action of furazolidone when fed at low levels is not precisely the same in the female as it is in the male bird. If it affected oögenesis as it does spermatogenesis, one would at least expect a delay in the onset of egg production and this did not occur. The slight decrease in weight gain in the group fed the drug at the 0·02 per cent level was of no statistical significance. Comb and wattle development appeared normal.

Cooper and Skulski (1955, 1956) showed that macro- and microscopic lesions occur in S.C.W.L. cockerels fed the drug at levels to 0·02 per cent, but did not continue the investigation to determine whether birds fed the drug at this level were capable of fertilizing hens. Moore, Chamberlin and Carter (1954) found no reduction in fertility when turkeys were fed furazolidone. If the drug produces no adverse effect in the female, as our results suggest, and does not always affect the male bird, fertility in large flock-matings may not always decline.

CONCLUSIONS

Furazolidone was fed to S.C.W.L. pullets at levels of 0, 0·01, 0·015 and 0·02 per cent during the period 14 to 26 weeks of age. Egg production was recorded for 24 weeks and fertility and hatchability for 13 weeks. Continuous feeding of the drug at these levels during this period had no significant effect on body weight, food consumption, date of first egg, or egg production. Using untreated cockerels, fertility and hatchability were not adversely affected.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Miss P. Reynolds for her technical assistance, to Mr. J. A. Binstead for incubation of eggs and to Dr. C. Horton-Smith for his helpful criticism in the preparation of the manuscript.

Furazolidone was provided through the courtesy of Messrs. A. J. White Ltd., Coldharbour Lane, London.

REFERENCES

Cooper, D. M., and Skulski, G. (1955). *Vet. Rec.*, **67**, 541; (1956). *J. comp. Path.*, **66**, 299.

Moore, E. N., Chamberlin, J. D., and Carter, R. D. (1954). *Poult. Sci.*, **33**, 1072 (abst.).

Snedecor, G. W. (1946). *Statistical Methods*, 4th Ed. Iowa State College Press; Ames, Iowa.

[*Received for publication, February 21st,* 1956]

THE BLOOD GROUPS OF THE PIG

IV. The A Antigen-antibody System and Haemolytic Disease in New-born Piglets

By

R. F. W. Goodwin

Department of Veterinary Clinical Studies, University of Cambridge

and

R. R. A. Coombs

Department of Pathology, University of Cambridge

INTRODUCTION

Injections of crystal violet swine fever vaccine can stimulate the production of red cell iso-antibodies in pigs (Goodwin, Saison and Coombs, 1955). Under certain circumstances these red cell iso-antibodies may cause haemolytic disease in new-born piglets, but their specificities have not been determined and this knowledge must await further studies on the blood groups of the pig. With the aid of the naturally occurring anti-A, however, the A antigen can be recognized and the A-O system clearly defined.

In preliminary studies on this blood group system it was not uncommon to find unvaccinated group O pigs with a serum anti-A titre of over 1,000. It thus seemed a possibility that if these antibodies were present in high concentration in colostrum, they might produce haemolytic disease in new-born group A piglets unless there existed some special protective mechanism.

In order to see whether a high titre of anti-A could give rise to haemolytic disease, the problem was investigated stage by stage according to the sequence of events governing the transfer of maternal iso-antibody to the neonatal red cells. Firstly, it was necessary to see how the serum titre of anti-A varied among unvaccinated group O pigs and whether the titre changed greatly in individual breeding animals, particularly during pregnancy. Secondly, we required to know whether anti-A in the serum of sows passed into the colostrum and became concentrated there. Thirdly, whether anti-A was absorbed from the alimentary tract after ingestion or neutralized by the salivary and gastric secretions and, finally, whether anti-A was destructive to group A cells *in vivo*.

MATERIALS AND METHODS

Blood and milk samples. Blood samples from adult animals were collected and stored as described previously (Saison, Goodwin and Coombs, 1955). Milk samples were taken without artificial aids. Baby pigs were bled by cutting off the tip of the tail. Samples for red cells were taken into acid-citrate-dextrose solution and those for haemoglobin estimations were collected over dried heparin. In the study of the transfusion reaction, haemoglobinaemia was estimated with the pre-injection plasma sample as the blank, and for plasma bilirubin a slight

modification of the method of Patterson, Swale and Maggs (1952) was used.*

Gastric mucin and saliva samples from piglets. Gastric mucin was taken with a Pasteur pipette from the stomachs of new-born piglets after they had been killed.

It was difficult to get a uniform sample of saliva. Sometimes sufficient neat saliva could be collected, but most commonly the mouths were swabbed with cotton wool on a stick and the swabs then squeezed out into about 0·5 ml. of saline. In most cases the samples could be crudely graded according to the amount of saliva mixed with saline and this proved useful in interpreting the results. Further observations on the method of collecting saliva samples are contained in the results.

The gastric mucin and saliva samples were placed in a boiling water bath for 5 to 10 minutes soon after collection and then kept at —20°C. until required for testing. Before use they were centrifuged and the inhibition tests were made with the clear supernatant fluids.

Grouping of red cells and titration of anti-A. These procedures were performed using the indirect anti-globulin sensitization test as described earlier (Saison *et al.*, 1955). After exposure to the anti-A serum the cells were washed three times (centrifuged four times) before the addition of the anti-globulin serum.

Demonstration of soluble A-substance in saliva or gastric mucin. Only certain dilutions of saliva or gastric mucin were tested with saline as a control (Table 4). To 0·1 ml. of these dilutions was added 0·1 ml. of a standard anti-A serum diluted 1 in 10 so as to contain 100 antibody doses. The mixtures were allowed to stand for ½ hour at room temperature. During this time, if A-substance is present the antibody is neutralized. The further procedure consisted of the usual anti-globulin technique to test for free anti-A. Thus 0·1 ml. of a 2 per cent suspension of group A cells was added to each tube and after a further incubation period of ½ hour at room temperature the cells were washed and examined for sensitization by rabbit anti-pig globulin serum. Absence of agglutination indicated the presence of soluble A-substance. With each batch of unknowns, a known group A and group O mucin sample were added as controls.

RESULTS

Investigations Concerned with Anti-A in the Sow

The level of anti-A in the serum of unvaccinated group O pigs.

The fifty-nine unvaccinated group O pigs examined in this small survey were from nine different herds and in age they ranged from six months to several years, so that probably all were old enough to have developed their natural antibody. Red cell iso-antibody reactive with pig A cells but not with our standard group O cell was accepted, for the purpose of this present work, as being anti-A. The standard group O cell had been selected during earlier work because it detected readily the iso-antibodies in a wide range of immune sera. The titres registered by the anti-globulin sensitization test are recorded in Table 1. It may be seen that there was a very big range in the levels of anti-A—from a titre of 1 to over 16,000.

*Both estimations were made through the kindness of Dr. John Marks.

TABLE 1

TITRE OF ANTI-A IN GROUP O PIGS

Titre of anti-A in the serum of fifty-nine unvaccinated pigs from nine herds.

Herd	*Number of pigs bled*	*Number of group O pigs having an anti-A titre of:*														
		<2†	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*1T*	*2T*	*4T*	*8T*	*16T*
Herd A	13	3	—	1	2	4	1	—	2	—	—	—	—	—	—	—
Herd B	2	—	1	—	1	—	—	—	—	—	—	—	—	—	—	—
Herd C	10	—	—	1	—	3	—	4	1	—	1	—	—	—	—	—
Herd D	4	—	—	—	—	1	1	1	—	—	1	—	—	—	—	—
Herd E	3	—	—	—	—	1	—	—	—	—	—	—	1	1	—	—
Herd F	11	2	—	—	—	1	1	—	—	5	1	1	—	—	—	—
Herd G	5	3	—	—	—	—	—	1	1	—	—	—	—	—	—	—
Herd H	8	—	—	—	—	—	—	—	1	—	—	1	3	—	2	1
Herd J	3	—	—	—	—	—	—	—	—	—	1	1	—	—	—	1*

*This titre was greater than 16,000 †This column includes four pigs where the serum titre was 1. T = Thousand.

No serious attempt was made at the time these examinations were performed to correlate the antibody levels with any particular state of the animals but on analysing the results no one factor appeared to be obviously related to a high level of anti-A. Thus the sample included animals of both sexes, in the ratio of two females to one male, and of varying ages; six breed types (Essex, Wessex, Welsh, Large White, Essex x Large White and Middle White x Large White); unmated, primiparous and multiparous animals: none of these variables correlated with the anti-A titre. In only one respect, and this is apparent in Table 1, was there a hint of a common influence as shown by a tendency for samples obtained from individual farms to have similar titres. Thus herd C had titres in the medium range (8 out of 10 being between 16 and 128), herd F showed high titres (7 out of 11 being between 256 and 1024), while herds E, J and H had very high titres (11 out of 14 being greater than 1,000). This suggests some environmental influence, particularly with herd H. Although the sample of 8 females represented two breeds and included 4 animals that were heavily pregnant and 4 that were unmated, the anti-A titres were almost uniformly high and they declined considerably on transferring the animals to our own farm (Table 2). It is difficult to reconcile these thoughts, however, with the wild fluctuations in antibody titre shown by other animals maintained here (Fig. 1).

It is possible that a larger sample of pigs, selected for particular factors, might uncover the cause of the great variation in the titres of this antibody but the point we were primarily interested in was that some group O pigs had a very high level of what appeared to be naturally occurring anti-A in their serum. If this antibody existed at a comparably high level in colostrum, then one of the conditions would have been satisfied for the production of haemolytic disease in the new-born due to anti-A.

Variation of anti-A titre in individual pigs

That the level of anti-A is by no means constant in one individual was first observed by subsequent examination of the eight pigs from herd H. Two further bleedings at three-monthly intervals showed that a great variation in the titre of antibody had occurred over a short period (Table 2). It followed from this that pigs could not be divided into two groups depending on whether they had a high anti-A titre or not, and that any risk at farrowing could be determined only by examinations made at the end of gestation.

Although the preliminary survey on the levels of anti-A among pigs in the field had indicated that pregnancy was not a prime cause of the variation in titre, it was nevertheless judged worthwhile to record the influence of pregnancy on the anti-A level of group O gilts when they had been mated to a group A boar. To this end, five such gilts, known to possess anti-A in medium concentration, were bled at regular intervals over a period of nine months during

TABLE 2

INCONSTANCY OF ANTI-A LEVEL

Fluctuation in the serum level of anti-A in eight unvaccinated pigs from herd H.

Herd H	*Titre of anti-A on*		
	18/10/54	*14/1/55*	*5/4/55*
Pig 10	2,000	256	512
„ 11	8,000	1,000	Dead
„ 12	16,000	<64	32
„ 13	128	256	64
„ 14	1,000	<16	1
„ 15	8,000	128	128
„ 16	2,000	<16	128
„ 17	2,000	64	32

which time they were in-pig to a group A boar and delivered their litters. Further evidence was obtained for the great variation of anti-A titre in a single animal, the fluctuations being so violent and variable that they exhibited no constant trend or pattern (Fig. 1). Thus in one animal the titre altered from 32 to 32,000 in four weeks or less, while the period close to farrowing was the only time at which the titres showed any marked parallel behaviour, for all five gilts then demonstrated a fall of titre and four out of the five showed a sharp rise in titre thereafter. But as all the gilts farrowed within a period of ten days, some other common factor could have been influencing antibody production at that time. It would be of great interest to determine the factors responsible for these variations. The possibility that migrating ascaris larvae might be involved is receiving attention.

The titre of anti-A in colostrum

Samples of colostrum were taken from group O unvaccinated pigs at or shortly after farrowing and the levels of anti-A were compared with those found in serum samples taken at the same time (Table 3). The samples from sows F12 and F10 (second farrowing) were taken within three hours of the birth of the last pig while those from gilt 52 and sow F10 (first farrowing) were obtained about six hours after farrowing. Gilts 57, 60 and 66 were grossly abnormal at farrowing and most of the piglets were savaged by their sows. Hence all the samples from these three gilts were obtained several hours after farrowing and the lack of normal nursing behaviour and suckling renders it difficult to appraise the titres for these animals.

It can be seen that anti-A, like other antibodies, appears in the colostrum and in some animals the concentration is such that, theoretically and in the absence of any protective mechanism, haemolytic disease could occur in new-born group A piglets.

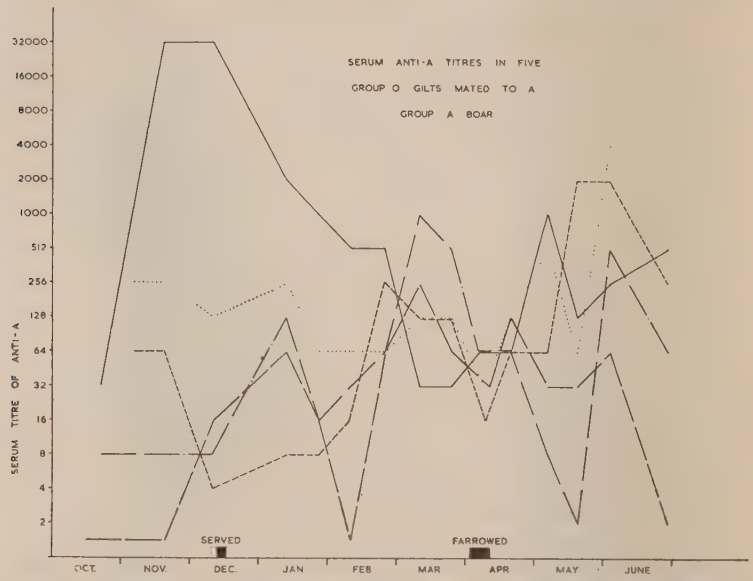


Fig. 1. Variations of anti-A titre shown by five group O gilts over a period of nine months. From December until April the gilts were in-pig to a group A boar.

Investigations Concerned with the Passage of Anti-A into the Piglet

Antibody is usually absent from the serum of the new-born pig but is obtained in large quantity from the colostrum during the first day of life by absorption from the gut. It was presumed that, in the absence of any special conditions, anti-A would behave similarly.

Development of the A antigen on the red cells of new-born piglets

During the investigations on the new-born an unexpected finding was encountered. On testing the cord bloods of a litter born from a group A x group A mating (first pregnancy, Table 4), it was found that the cells of all the piglets failed to react with anti-A serum and this led us to suspect that in the pig the A antigen might be absent at birth. To investigate the development of this antigen on the red cells, therefore, the litters from pregnancies 2, 5, 6 and 7 were used (Table 4). Cord blood samples were taken at or shortly after birth and further bleedings made at frequent intervals, usually

TABLE 3

ANTI-A IN COLOSTRUM

Titre of anti-A in samples of colostrum and serum taken together.

Pig	*Titre of anti-A*	
	Serum	*Colostrum*
Sow F12	16,000	16,000
„ F10	2,000	512
„ F10 (b)	256	16,000
Gilt 52	32	128
„ 57	64	256
„ 60	16	<4
„ 66	32	4

b=subsequent pregnancy

every 3 or 4 days, during the first month of life. These cell samples were tested with anti-A serum using the anti-globulin technique. The change in the serological behaviour of the cells of the new-born pig—from a failure to react with anti-A to the normal reaction shown by the adult group A cell—is depicted in Table 4.

It may be seen that in all the piglets (65 in number) the cord red cells failed to react with anti-A serum. However, of the 37 animals that survived 23 were of group A by later red cell tests and, from the saliva and mucin tests, there were probably at least a further 12 group A animals among those that were killed or died. Thus the absence of the red cell antigen at birth was confirmed.

Although the A antigen was adumbrated as early as the fifth day in some pigs (sooner perhaps had the tests been made) the earliest clear and strongly positive reaction was seen at the 10th day. On the other hand some piglets did not yield strongly positive tests until about the 30th day. This marked variation in the rate of appearance of the A antigen can be seen even among litter-mates.

In the early phases of this development of the A antigen, the microscopic readings of the agglutination tests show small tightly agglutinated clumps of cells in a sea of unagglutinated cells. Gradually the proportion of agglutinated to unagglutinated cells increases, until eventually all the cells are agglutinated to some degree or other. Such a change in appearance suggests that cells lacking the A antigen are gradually replaced by fully reactive group A cells. It is interesting that the red cells of the new-born piglet exhibit a marked change in carbohydrate metabolism after birth (Goodwin, 1954) and that this change, also believed to be due to

the influx of a new type of cell into the circulation, is likewise well-advanced when the piglet is about one month old.

The fact that the A antigen does not appear until after birth and is not fully developed until the latter part of the first month of life is inconsistent with the possibility that this antigen-antibody system could give rise to haemolytic anaemia in new-born piglets.

TABLE 4

DEVELOPMENT OF THE A ANTIGEN

Development of reactive A antigen on red cells and detection of soluble A-substance in saliva and gastric mucin.

Mating	Piglet	Reaction with anti-A on day: *1	4		10	14	17	24	110	Presumptive group from reaction of red cells	†A-substance in gastric juice at birth	†A-substance in saliva at birth	†A-substance in saliva fifth day	Presumptive group on evidence of soluble A-substance
1. (Group A × Group A)	1	—	D									>100		A
	2	—	D									<1,000		
	3	—	D								>1,000 <10,000			A
	4	—	D								>1,000 <10,000			A
	5	—	D									>100 <1,000		A
2. (Group A × Group A)	1	—	—		w	++	++			A				
	2	—	—		—	—	a	a	—	O				
	3	—	—		—	—	a	a	—	O				
	4	—	—		—	+	++			A				
	5	—	—		+	++	++			A				
	6	—	—		w	D								
	7	—	—		—	+	++			A				
	8	—	—		—	D								
	9	—	—		—	w	+	++		A				
	10	—	—		—	a	a	a	—	O				
	11	—	D								<10	<10		O
	12	—	D								>100 <1,000	>10 <100		A
	13	—	D								>1,000 <10,000	>100 <1,000		A
	14	—	—		++	++	++			A				
3. (Group O × Group O)	1	—							—	O				
	2	—							—	O				
	3	—							—	O				
	4	—							—	O				
	5	—	D								<10	<10		O
	6	—	D								<10	<10		O
4. (Group O × Group O)	1	—	D								<10	<10		O
	2	—	D								<10	<10		O
	3	—	D								<10	<10		O
	4	—	D								<10	<10		O
	5	—	D								<10	<10		O
		*1	4	8	12	15	19	27	45					
5. (Group A × Group A)	1	—	D									<10		O
	2	—	D									>50		A
	3	—	—	—	w	+	++	++	++	A		>50	>50	A
	4	—	D									>5 <20		?
	5	—	—	—	+	++	++	++	++	A		>50	>50	A
	6	—	D									>50		A
	7	—	—	—	—	a	a	—	—	O		<5	<5	O
	8	—	—	—	—	a	a	—	—	O		>5 <50	<5	O
	9	—	D									>20 <80		A

*The day of birth is called ' Day 1 ', and so on.

D = Dead.

†Figures show dilution of saliva or mucin inhibiting the sensitizing power of 100 doses of anti-A.

—, a, w, + and ++ = Degrees of agglutination of cells by anti-globulin serum after exposure to 100 doses of anti-A (— = No agglutination; a = Not completely negative clumps in a negative field).

TABLE 4—*Continued.*

DEVELOPMENT OF THE A ANTIGEN

Development of reactive A antigen on red cells and detection of soluble A-substance in saliva and gastric mucin.

Mating	Piglet	Reaction with anti-A on day: 1*	5	8	12	15	18	22	26	29	33	54	Presumptive group from reaction of red cells	†A-substance in saliva on day 1	5	Presumptive group on evidence of soluble A-substance
6. (Group O × Group A)	1	—	—	—	—	a	—	—	—	—		—	O	weak 5	<5	O
														<50		
	2	—	w	+	++	++	++	++					A	500	>500	A
	3	—	—	w	w	w	w	w	+	+	+	++	A	>50	>500	A
														<500		
	4	—	D											>500		A
	5	—	w	w	w	+	w	+	++	++	++		A	>50	>500	A
														<500		
	6	—	D											>50		A
														<500		
	7	—	—	—	—	a	—	—	—	—		—	O	<5	<5	O
	8	—	D											<5		O
	9	—	—	—	w	w	w	w	+	++	++	++	A	>50	>500	A
														<500		
	10	—	—	w	w	+	++	++					A	>50	>500	A
														<500		
	11	—	—	w	w	++	++	++					A	>50	>500	A
														<500		
	12	—	D											weak 5		?
														<50		
	13	—	—	—	—	a	—	—	—	—		—	O	>5	>5	?
														<50	<50	
		1	5	7	10	14	17	20	24	28	31	56				
7. (Group A × Group A)	1	—	—	—	+	++	++	++	++				A	250	>500	A
	2	—	—	—	w	++	++	++	++				A	250	>500	A
	3	—	D											>25		A
														<250		
	4	—	—	—	w	++	++	++	++				A	>250	>500	A
	5	—	w	—	w	++	++	++	++				A	>250	>500	A
	6	—	—	—	w	++	++	++	++				A	>250	>500	A
	7	—	—	—	—	—	a	—	—	—	—	—	O	<2·5	<5	O
	8	—	—	—	—	—	a	w	+	++	++		A	>25	>500	A
														<250		
	9	—	—	—	—	—	w	+	+	++	++		A	>250	>500	A
	10	—	—	—	—	—	w	+	+	++	++		A	>250	>500	A
	11	—	—	—	—	—	—	a	—	—	—	—	O	<2·5	<5	O
	12	—	—	—	—	—	a	+	+	+	++	++	A	>250	>500	A
	13	—	D											<2·5		O

*The day of birth is called ' Day 1 ', and so on.

D = Dead.

†Figures show dilution of saliva or mucin inhibiting the sensitizing power of 100 doses of anti-A. —, a, w, +, and ++ = Degrees of agglutination of cells by anti-globulin serum after exposure to 100 doses of anti-A (— = No agglutination; a = Not completely negative: clumps in a negative field).

A-substance in the saliva and gastric juice of new-born piglets

Various piglets from the seven litters shown in Table 4 were examined for A-substance in their secretions. In the first pregnancy, gastric mucin was obtained from two piglets and saliva from another two. In the second, third and fourth pregnancies, however, saliva and gastric juice were obtained together. As the collection of gastric mucin necessitated killing the piglets and this prevented any subsequent correlation between the inhibition tests and the development of the A antigen on the red cells, examinations for soluble A-substance in the fifth pregnancy were confined to saliva samples. These were collected at birth and again on the fifth day of life. The later samples were added because mouth swabs taken at birth were easily contaminated with blood, amniotic débris etc. By the sixth

and seventh pregnancies it was possible to profit from several experiments on sampling technique and the saliva samples were generally much more satisfactory. Thus, by allowing the piglets to suckle for several hours after birth and thoroughly wash their mouths with colostrum, all gross contamination was eliminated, and sampling late on the first day of life, instead of at birth, became the routine procedure for all piglets in these last two pregnancies.

A specific inhibition of anti-A serum by a saliva or mucin sample diluted 1 in 10 or more was accepted as indicating the presence of A-substance. As an indication of the amount of A-substance in gastric mucin it may be said that a dilution between 1 in 1,000 and 1 in 10,000 was sufficient to inhibit an equal volume of anti-A serum containing 100 agglutinating doses. The amount in saliva appeared to be less but the estimations cannot be readily compared as the samples were collected in such different ways.

The remainder of this section summarizes and discusses these investigations on the soluble A-substance; the detailed results are given in Table 4 where they are more conveniently compared with the red cell tests.

In group O x group O matings no A-substance could be demonstrated in the saliva or gastric mucin of the piglets. On the other hand in group A x group A matings, A-substance could be shown in both these secretions at birth. Not all the piglets from the latter matings, however, had soluble A-substance in their secretions but all such deficient piglets that survived from the last three pregnancies proved to be of group O by red cell tests. Nevertheless, it is possible that not all group A pigs secrete the A-substance and this question still requires investigation.

Altogether, 24 piglets were examined for both the presence of A-substance in the saliva and the development of the A antigen on the red cell. Of these, 17 were of group A and 7 of group O. In all but three animals the presumptive grouping by saliva tests at or shortly after birth proved to be the same as the final red cell grouping. The exceptions were all group O pigs in which there was weak inhibition with the first-day saliva samples. In two of the exceptions (no. 8, fifth pregnancy and no. 1, sixth pregnancy) the saliva samples taken on the fifth day were negative and showed the greater reliability of later sampling, but in the remaining piglet (no. 13, sixth pregnancy) the fifth-day sample remained doubtful even on repeating the test. Furthermore, by the 54th day, when a third saliva sample from this particular pig was obtained, the test was still not unequivocal: although a 1:5 dilution of this saliva sample did not completely inhibit the anti-A serum, the resulting red cell agglutination was noticeably weaker than that obtained with the serum previously treated with control (group O) saliva. However, this result would not be confused with the result obtained with saliva from a group A piglet. Tests were not made to see

whether these anomalous weak inhibitions were specific for the A antigen.

It is apparent from these results that, in all probability, any ingested anti-A would be neutralized by the A-substance present in the alimentary secretions of the new-born group A piglet. Even though the red cells lack the A antigen for some days after birth, such neutralization might still be important during the neonatal period.

Advantage can be taken of the presence of the A-substance in these secretions to group new-born piglets at a time when this cannot be done by red cell tests. However, it must be borne in mind that both such grouping and the physiological neutralization of maternal anti-A would fail in any group A piglet that did not secrete the A-substance.

Absorption of anti-A by the new-born piglet

It seemed probable from the results in the preceding section that the A-substance secreted by group A piglets would prevent the absorption of anti-A from the gut. To see if this were so, it was necessary to examine the sera of new-born piglets after they had suckled this antibody in colostrum under natural conditions. Ideally, we wished for sows with a high serum titre at farrowing so that anti-A would be abundantly available and, by having such sows in-pig to group A boars, we hoped for a sufficient number of group O and group A piglets in each litter to demonstrate conclusively that anti-A passed into the circulation of only the group O animals. However, although high titres of anti-A had not been uncommon in this work, during the last stages of the investigation there were no sows available with high levels of anti-A at term and two sows with low titres were studied instead. The result proved inconclusive.

The first sow had an anti-A titre of 32 six days before farrowing. However, colostrum taken at the beginning of farrowing (after the birth of pig 3) contained anti-A at a titre of only 4 to 8. As only four pigs were born in $3\frac{1}{2}$ hours, the sow was left to continue farrowing undisturbed. No more pigs had been born after a further 8 hours and during the next hour 12 pigs were removed manually. Colostrum taken after the last pig had been delivered showed only a weak reaction for anti-A when tested undiluted. Thirteen piglets were still alive approximately 24 hours after the beginning of farrowing and they were each, then, bled for serum. No anti-A could be detected in any of these serum samples. Four of the manually delivered pigs were probably of group O (by saliva tests) but only two survived for this grouping to be confirmed by red cell tests. It might be expected that anti-A would be at undetectable levels in these four piglets when the antibody was at such low concentration in the colostrum at the time of their birth. One of the first four piglets born (pig 2) was also a group O (by saliva and red cell tests)

but although it had greater opportunity for ingesting anti-A, the total amount of this antibody in the gut was probably still small.

The second sow had a serum titre of 128 three days before farrowing. Colostrum taken at the birth of the second piglet contained anti-A at a titre of 16. Parturition was normal and three of the seven piglets born were of group O by later red cell tests. However, the sera of all the piglets were negative when tested for anti-A at 20 hours and, again, at 38 hours of age. It would not have been surprising if anti-A had been found in the circulation of these group O piglets. On the other hand, the colostral titre was sufficiently low when farrowing commenced for us to question whether the total quantity of antibody ingested was, once again, insufficient for our purposes.

These experiments will be repeated at the earliest opportunity using sows with higher serum titres at farrowing. In the absence of satisfactory results with piglets that ingested anti-A by the natural route, the antibody has been introduced by injection.

Three new-born piglets (one of group A by saliva tests and two of group O) were prevented from suckling at birth and removed to the laboratory for the duration of the experiment. All were injected intraperitoneally with 10 ml. of a serum that contained anti-A at a titre of 512. Serum samples taken from all the animals before injection were free of anti-A. In the group O piglets, anti-A was detected 10 hours after injection at a serum titre of 4 in one animal and 8 in the other. In the group A piglet, however, serum samples taken 2 and 10 hours after the injection were negative for the antibody. This suggests that fixed or soluble A-substance exists elsewhere than in the saliva and gastric juice, and thereby neutralizes anti-A before it can reach the circulation.

Interaction of Group A Pig Cells and Anti-A in vivo

Although it seemed highly probable that the interaction of anti-A and group A red cells in the body would give rise to a haemolytic or other shock reaction we felt that this should be confirmed. The fact that pig anti-A acts as a very poor haemolysin *in vitro* with pig, guinea-pig or rabbit complement, and does not agglutinate directly pig A cells, except in very high concentration and in neat serum, suggested that the reaction might not be very dramatic.

It was obviously not possible to observe this reaction in very young pigs and, as a first experiment, two group A pigs, aged 6½ weeks, were injected intravenously with 40 and 60 ml. respectively of a pig anti-A serum having a titre of 1,000. In neither case were any clinical signs observed. When the blood of the pig receiving 40 ml. was examined 1, 4 and 24 hours later there was no evidence of a fall in haemoglobin nor did the red cells give a positive direct anti-globulin test. The blood of the pig receiving 60 ml. gave only

a very weak direct anti-globulin test and again there was no evidence of a fall in the haemoglobin level. On the contrary, both pigs showed a slight rise in haemoglobin concentration, probably because they had missed several feeds. It was then decided to perform a reciprocal experiment and inject group A blood into group O pigs.

A sow (approx. 240 lb. weight) with a serum anti-A titre of 32 was injected intravenously with 100 ml. of citrated group A blood (1 part acid-citrate-dextrose solution and 2 parts blood). The injection was given quickly over a period of 3 minutes. No clinical signs were observed. Blood samples were taken into bottles containing dried heparin before the injection and 1, 3 and 4½ hours later. No haemoglobinaemia or bilirubinaemia developed over this time. The figures for the bilirubin estimations were all within the range of 0·2 to 0·3 mg. per 100 ml. of plasma.

Another sow (approx. 400 lb. weight) with an anti-A titre of 256 was injected intravenously with 165 ml. of the same citrated group A blood. The first 65 ml. was infused in 1½ minutes. The pig collapsed to the ground and showed a purple discolouration of the ears and mammary glands. Within 2 minutes the pig got up and a blood sample was taken. The infusion of group A blood was continued and another 100 ml. was injected. Within 3 minutes the pig became unsteady again and the purple discolouration spread over the whole body. However, the colour quickly disappeared and in 5 minutes from the second injection nothing abnormal was noticed about the animal. Further heparinized blood samples showed that a haemoglobinaemia developed but no significant change in the plasma bilirubin level could be detected 2 and 3½ hours after the reaction (Table 5). Both pigs appeared quite normal clinically when observed over the following days.

TABLE 5

TRANSFUSION REACTION

Haemoglobinaemia and bilirubinaemia following the intravenous injection of 165 ml. of group A blood into a sow having a serum anti-A titre of 256.

Sample	*Haemoglobinaemia g./100 ml. plasma*	*Bilirubinaemia mg./100 ml. plasma*
Pre-infusion	nil	0·2
After first reaction ..	0·02	0·1
2 hrs after reaction ..	0·07	0·25
3½ hrs after reaction ..	0·08	0·15

We conclude from this experiment that anti-A has a destructive action on A cells *in vivo* but that probably a great amount of antibody is necessary to produce severe haemolysis or shock. It may be,

however, that the pig is able to withstand well the stress of sudden intravascular haemolysis.

CONCLUSIONS

The A antigen-antibody system of the pig has been studied to see whether anti-A could give rise to haemolytic disease in new-born group A piglets.

Anti-A was detected in the sera of all but four of fifty-nine adult group O pigs; the four exceptions were not necessarily negative, however, as their sera were not tested undiluted. The level of anti-A varied greatly from pig to pig (between titres of 1 and 32,000). Great variations in anti-A titre also occurred in individual animals over a short period of time so that group O pigs could not, except very temporarily, be divided according to the serum titre of the antibody. There is at present no explanation for these rapid changes of titre.

Anti-A passed into the colostrum and was often concentrated there. New-born pigs born of sows with high anti-A titres, therefore, ingested the antibody in high concentration.

The A antigen is absent from the red cells of new-born group A piglets but it appears in increasing strength at varying times during the first month of life. This fact is inconsistent with the possibility that anti-A could produce haemolytic anaemia in the new-born.

The saliva and gastric juice of new-born group A piglets (but not group O piglets) contained soluble A-substance which could neutralize anti-A. In the absence of the red cell antigen at birth, piglets can be grouped with a high degree of accuracy by testing saliva or gastric secretions for soluble A-substance. It is not known, however, whether all group A piglets secrete the A-substance.

The injection of group A cells into two adult pigs possessing anti-A in their serum resulted in a mild haemolytic reaction, with haemoglobinaemia, in one of the animals where the anti-A titre was the higher (256). Clinically the reaction was soon over and there were no obvious after-effects.

ACKNOWLEDGMENTS

The experience of Mr. R. H. M. Matthews with the serological techniques employed and his outstanding reliability in their performance allowed us to undertake this investigation concurrently with much other work on the pig. Most of the titrations that were necessary for the preliminary field survey of anti-A titres were performed by Miss Ruth Saison. Mr. P. J. D. V. Brett has assisted in all the work associated with the farrowings and the collection of biological samples. One of us (R.R.A.C.) would like to express his thanks to both the Agricultural Research Council and the Nuffield Foundation for a joint personal grant. This work is part of a wider study that has been encouraged by Professor L. P. Pugh and aided financially by the Agricultural Research Council.

REFERENCES

Goodwin, R. F. W. (1954). *Nature, Lond.*, **173**, 777.
Goodwin, R. F. W., Saison, R., and Coombs, R. R. A. (1955). *J. comp. Path.*, **65**, 79.
Saison, R., Goodwin, R. F. W., and Coombs, R. R. A. (1955). *Ibid.*, 71.
Patterson, J., Swale, J., and Maggs, C. (1952). *Biochem. J.*, **52**, 100.

[*Received for publication, February 22nd,* 1956]

STUDIES ON *CORYNEBACTERIUM RENALE*

V. Some Observations on a Chemical Extract

By

R. Lovell

Department of Pathology, Royal Veterinary College, London

INTRODUCTION

Corynebacterium renale is known to be pathogenic for cattle under certain ill defined natural circumstances and to produce kidney lesions in a proportion of mice and rabbits after intravenous inoculation (Lovell, 1946; Lovell and Cotchin, 1946; Feenstra, Thorp and Gray, 1949). Attempts have been made to relate the disease-producing capacity of the organism to chemical extracts of the bacterium; brief references have been made to the effect produced in mice by such extracts (Lovell, 1951; 1953 a. & b.) but no details have been reported. An account is given of the method of preparation of a chemical extract and of some of the properties which it exhibits.

METHODS

Bacterial extracts. Extracts were prepared in a manner similar to that used by Middlebrook and Dubos (1948) for tubercle bacilli. A standard strain of *C. renale* is grown on agar at 37°C for 48 hours and the growth washed off with saline and glass beads. The thick suspension is treated with 10 volumes of cold acetone which, after stirring, is filtered through paper and the deposit collected and dried in a desiccator; after 3 to 4 days the powder is ground up in a mortar.

Two grams of the acetone-treated product is added to 50cc of 80 per cent phenol and the mixture stirred for 20 hours at 37°C; after centrifugation the deposit is treated with another 50cc quantity of phenol and stirred again for 20 hours at 37°C. This is centrifuged, the deposit washed three times in cold acetone and dried in a desiccator. The dry powder is ground in an agate mortar and 0·5g. is added to 65cc of a solution containing:—0·55 per cent NaCl, 0·5 per cent Na_2HPO_4 and 20 per cent methanol. The mixture is stirred for 20 hours at 37°C after which it is centrifuged for one and a half hours and the supernatant fluid is collected and the pH adjusted to 6·0. This solution is dialysed in a cellophane bag against running water for four days and centrifuged to remove any granules or deposit. The supernatant fluid is collected, the pH adjusted to 6·5 to 7·0, and rendered isotonic by the addition of 0·52cc of saturated salt solution to every 20cc.

Serological reactions. Precipitin tests were made with the extract and sera prepared by immunisation of rabbits with formolised suspensions of *C. renale.*

Agglutination tests were made by mixing sensitised red blood corpuscles of sheep and diluted sera. Washed centrifuged red cells were sensitised by adding them to the fluid chemical extract in the proportion of 0·5 cc of a thick suspension of red cells to 10 cc of extract. The mixture was kept at 37°C for 2 hours during which time it was shaken frequently;

it was washed in saline three times and after the final centrifugation the supernatant fluid was discarded and the sensitised red cells made up to the original volume which in this case was usually 100 cc.

Serum dilutions were made in 1 cc quantities and varied from a primary dilution of 1:5. Equal quantities of sensitised red cells were added and, after mixing, the tubes were incubated in a water bath at 37°C for 30 minutes and were examined. Further readings were taken after 2 hours at room temperature and usually on the following day. A control tube of 1 cc of serum diluted 1:5 and 1 cc of washed normal sheep cells was always included since some normal sera may agglutinate normal sheep cells.

Demonstration of effect of extract on kidneys of mice. Mice of about 18 g. weight were anaesthetised by the intravenous injection of 0·0015 g. of nembutal. The kidneys were exposed when the mice were fully under the influence of the nembutal and the extract was given intravenously. Observations were made and on some occasions an injection of india ink or similar substance was made shortly afterwards and by the same route.

OBSERVATIONS AND RESULTS

Chemical Tests

Limited observations were made on the chemical nature of the extract of *C. renale*. In solution it is fairly opalescent and gives a positive reaction with Molisch's reagent, indicating the presence of carbohydrates. It decomposes urea indicating the presence of urease and a reaction is obtained with Millon's reagent. It therefore appears to be mainly a carbohydrate. Lister (personal communication) hydrolysed 0·5 g. of a freeze-dried preparation in 1 cc. of 3 per cent nitric acid at 100°C. for 1 hour, added 70 mg. of barium carbonate and freeze-dried the preparation. The product was extracted with absolute alcohol and when subjected to chromatographic analysis in phenol-ammonia and pyridine-ethyl acetate-water it was possible to identify ribose, arabinose, mannose and galactose in about equal quantities.

Serological Tests

Precipitin. Precipitation occurs between immune serum prepared against a formolised suspension of *C. renale* and the extract, whether the latter is heated at 56°C. for half an hour or not.

Agglutination of sensitised red cells. Agglutination of sheep red cells sensitised to the extract also occurs when mixed with immune serum. Preliminary observations showed that the optimal concentration of sheep red cells in these tests was half that found in normal blood and this was used accordingly.

A number of normal bovine sera were examined for agglutinins against sensitised sheep red cells. Equal quantities of sera, diluted from 1:5 to 1:640, were mixed with sensitised red cells. The tests were controlled by the inclusion of mixtures of normal sheep red cells and each bovine serum at a 1:5 dilution; immune rabbit serum was titrated with each batch of bovine sera examined. Specific agglutination was not accepted unless the normal cells were

not agglutinated by the bovine sera and the sensitised cells were agglutinated by the immune rabbit serum to dilutions of 1:640 or 1:1280. The results obtained were, therefore, specific and constant, and they are given in the form of frequency distributions in Table 1.

TABLE 1

RED CELL AGGLUTINATION

Number of sera of cattle of different ages which agglutinated sheep red cells which had been sensitised with *C. renale* extract.

Dilutions	*Calves (from birth to six months of age)*				*Heifers*	*Oxen, Cows*	*Total of all ages of cattle*
	Up to 1 week	*2 – 4 weeks*	*Exact age unknown*	*Total number*	*2 – 3 years of age*	*5 years and over*	
0	13 (22·0)	41 (58·5)	3	57 (39·3)			57 (27·0)
1 : 10	23 (39·0)	18 (25·7)	1	42 (29·0)		1	43 (20·5)
1 : 20	20 (34·0)	9 (12·8)	2	31 (21·4)		1	32 (15·2)
1 : 40	3 (5·0)		1	4 (2·7)			4 (1·9)
1 : 80		2 (2·8)	4	6 (4·1)	1	10 (23·3)	17 (8·1)
1 : 160			3	3 (2·1)	7 (30·4)	10 (23·3)	20 (9·5)
1 : 320			1	1	12 (52·2)	12 (28·6)	25 (11·9)
1 : 640+			1	1	3	8 (19·0)	12 (5·7)
Totals	59	70	16	145	23	42	210

The numbers in parenthesis are percentages which are given only when the total numbers appear adequate.

Normal bovine sera contain antibodies against *C. renale* and in the total column of Table 1 there are two peaks, one at the lower end of the scale and a small one at the 1:160 to 1:320 dilutions. An analysis of these shows that in very young calves there is a peak at 1:10 to 1:20 and this decreases in the early weeks of calfhood. Cattle of two years and upwards show a peak at the higher dilutions of 1:160 to 1:640.

These results suggest that the antibodies in the sera of the very young calves may have been obtained from their mothers and that the decrease in frequency of the low titres in the older calves is because the antibodies are passive ones. The antibodies in the sera of adult cattle are probably active in nature and possibly associated with the common occurrence of *C. renale* in normal healthy cattle (Weitz, 1947).

This chemical extract is clearly of some immunological significance and would appear to be related to the surface of the bacterium. This is supported by the fact that immune sera prepared against whole bacteria reacted as a precipitin and as an agglutinin of

sensitised red cells; furthermore it agglutinates whole organisms in a concentration of salt outside the range of sensitivity of *C. renale.*

Effect of the Extract on the Kidney

The intravenous injection of the chemical extract into mice produces a marked effect upon the kidneys. Within a few seconds of the injection the kidneys become pale, blanched and bloodless as if a spasm of the renal artery had prevented the blood from reaching the organ. Some of the observations seemed to suggest that the ischaemia was confined to the cortex, but it has not been possible to demonstrate this consistently. Had it been, it would suggest that the blood is being diverted through the juxtamedullary glomeruli giving rise to a cortical ischaemia comparable to that described by Trueta, Barclay, Daniel, Franklin and Prichard (1947). The picture is not so clear cut as this and the whole of the kidney appears to be affected as is shown in the accompanying figures. The blood supply of other organs such as liver and spleen is not affected and this vascular difference may be accentuated by injecting india ink intravenously immediately after the blanching of the kidney has been observed. The india ink is diffused throughout the other organs, leaving the kidneys pale with little or no blackening.

A few experiments were made with extracts of other bacteria. Extracts of *C. equi* and staphylococci produce a similar effect, whereas extracts of *Bact. coli* do not.

DISCUSSION

The chemical extract which has been prepared from *C. renale* appears to be of some significance both from the pathogenic and serological angles. It has previously been shown that *C. renale*, when given intravenously to mice, will multiply in the kidneys (Lovell and Cotchin, 1946) and it was expected that this extract would induce a cortical ischaemia. This could lead to the hypothesis that the diversion of blood to the medulla is related to the multiplication of *C. renale* in that part of the kidney. This hypothesis cannot, at the moment, be supported because of the variations in the circulatory disturbance which occurs in the kidney after the intravenous inoculation of the extract. A loss of colour takes place within a few minutes of the injection and in some cases there was a suggestion of ischaemia of the cortex alone. This was not consistent and the question whether the phenomenon is similar to that induced by Trueta, *et al.* (1947) in rabbits by physical means and by staphylococcal toxin is at the moment an open one. The present findings are more akin to those of Thal (1955) who also studied renal vasospasm in rabbits induced by staphylococcal toxin. In comparing his results with those of Trueta, *et al.* (1947) he emphasises that a partial renal ischaemia was seen in relatively few of the animals studied. In general he showed a virtually complete

renal ischaemia resulting from the selective effect of the staphylococcal toxin on the renal blood vessels; the initial disturbance was probably a spasm of the venules or veins. Although multiplication of *C. renale* in the natural disease in the cow and in the experimental disease in mice takes place largely in the medulla it cannot be stated categorically that the chemical extract of the organism influences the blood flow in the kidney in such a way as to induce ischaemia of the cortex alone and diversion of the blood supply to the medulla. It is possible that further study of some of the factors involved such as the dosage and timing may enable one eventually to induce constant results with regularity; it is not possible at the moment.

The absorption of the extract on the red blood cells and the use of these sensitised cells for the testing of antibodies in sera is useful for survey work. The determination of the antibody content of a serum by its precipitin content is not sufficiently sensitive, because the bulk of the precipitate is derived from the antibody. Direct agglutination tests are difficult as suspensions of *C. renale* tend to become agglutinated by the action of heat alone and are unsuitable for routine work. Heat alone may induce sedimentation of suspensions made in glycerinated water and the salt sensitivity of *C. renale* usually ranged from 6·25 per cent NaCl to 0·2 per cent. Tests were made with suspensions which when mixed with sera contained 10 per cent NaCl. These were moderately satisfactory and agglutination by immune sera occurred, but the haemagglutination test described above appears to be more reliable, especially if a survey of antibodies in a normal population is being made. The limited survey which has been made suggests that antibodies against *C. renale* are common in normal cattle sera. It further shows that there may be a higher concentration in the sera of calves in the first week or two of life and that the waning of this may be related to the normal loss of maternal antibodies. The antibodies in the sera of older animals may be of an active nature. If this is the correct interpretation there is support for views already expressed that *C. renale* is carried by a high proportion of normal healthy cattle.

CONCLUSIONS

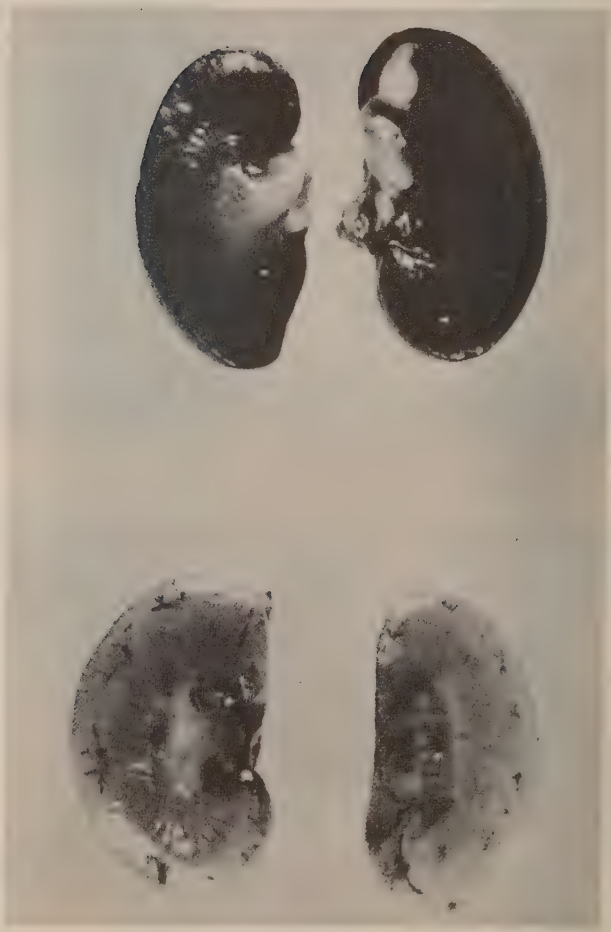
The preparation and properties of a chemical extract of *C. renale* are described. It appears to be mainly carbohydrate in nature, consisting of ribose, arabinose, mannose and galactose. It forms a precipitate with immune serum and may be absorbed on to the red blood cells of the sheep. The sensitised cells are agglutinated by immune sera and may be used for the demonstration of *C. renale* antibodies in normal sera. Intravenous inoculation of the extract into mice causes a disturbance of the circulation in the kidney which is demonstrated by a blanching of these organs with an unimpaired blood flow in spleen and liver. The pathogenic and serological significance of this extract is discussed.

Fig. 1

Fig. 2

To face page 336

Fig. 3



ACKNOWLEDGMENT

I am indebted to Mr. A. J. Lister of the Unit of Chemical Microbiology, Cambridge for details of the composition of the extract.

REFERENCES

Feenstra, E. S., Thorp, F., and Gray, M. L. (1949). *Amer. J. vet. Res.*, **10**, 12.
Lovell, R. (1946). *J. comp. Path.*, **56**, 196; (1951). *Vet. Rec.*, **63**, 645; (1953, a) *Proc. R. Soc. Med.*, **46**, 179; (1953, b) *VI. Int. Cong. Microbiol.*, **1**, 774.
Lovell, R., and Cotchin, E. (1946). *J. comp. Path.*, **56**, 205.
Middlebrook, G., and Dubos, R. J. (1948). *J. exp. Med.*, **88**, 521.
Thal, A. (1955). *Amer. J. Path.*, **31**, 233.
Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. M. L. (1947). *Studies of the Renal Circulation.* Blackwell; Oxford.
Weitz, B. (1947). *J. comp. Path.*, **57**, 191.

[*Received for publication, March 6th,* 1956]

Fig. 1. Blanching of kidneys in mouse after intravenous injection of chemical extract of *C. renale*. Blood supply of spleen and liver not affected.

Fig. 2. No change in blood supply of kidneys or other organs after injection of saline made under similar circumstances to that in Fig. 1.

Fig. 3. Kidneys of mouse injected intravenously with saline and india ink compared with kidneys of mouse injected with chemical extract of *C. renale* and india ink below; the renal ischaemia in the latter is almost complete.

STUDIES ON HOST RESISTANCE TO DICTYOCAULUS INFECTION

III. Experiments on the Site of the Protection Mechanism

By

J. F. Michel

Veterinary Laboratory, Ministry of Agriculture, Weybridge

INTRODUCTION

In an earlier paper it was shown that larvae administered to a lamb that had previously experienced infection failed to become established in the lungs. It seemed of interest to enquire into the site of this mechanism and into the fate of the larvae, earlier experiments having indicated that larvae failed to reach the lungs of a resistant animal.

The route by which *D. filaria* larvae migrate to the lungs was worked out by Hobmaier and Hobmaier (1929) who demonstrated that migration took place through the mesenteric lymph glands. Consideration of this route shows that having penetrated the lymphatics of the intestinal wall the larvae are transported mechanically by the lymph and subsequently by the blood and encounter only one obstacle, the mesenteric lymph glands, before being filtered out in the capillaries of the lungs. The following causes may explain the failure of larvae to become established in the lungs of a resistant animal. They may fail to penetrate the intestinal wall, or be destroyed there. They may be destroyed in the mesenteric lymph glands or fail to leave them. They may be carried right through the capillary system of the lungs, fail to break out of the blood vessels into the alveoli, or be destroyed in or eliminated from the air spaces of the lungs.

Preliminary observations on small animals showed not only that the route of migration of *D. filaria* larvae in small laboratory animals was the same as in sheep but also that previous experience of infection enabled these animals to resist the establishment of worms administered to them. Although in these unnatural hosts the worms failed to grow to maturity it was considered that results obtained with them might throw some light on the "protection" mechanism. They should however be interpreted with caution.

METHODS

Larvae from the lungs and lymph glands of sheep were recovered by means of the Baermann apparatus. The tissue was finely minced and immersed over a screen in saline at 37°C in a large funnel. The lungs and lymph glands of mice and rabbits were similarly treated in 4 in. funnels filled with saline and kept overnight in an incubator at 37°C. The minced tissue remaining after this process was, in the case of these small animals, subjected to peptic digestion. The

figures quoted represent the total number of larvae recovered by the entire procedure.

Larvae for oral administration were obtained and cultured to the third stage in the manner previously described (Michel, 1956). For intravenous administration, third stage larvae were first induced to complete their first and second ecdyses. A clean sample of larvae was suspended in 7 per cent. 'Milton' in a shallow dish and silver sand was added until there was no free fluid showing above the sand. After six hours at room temperature the process was complete and the larvae were washed out of the sand, thoroughly washed over a wire screen having 400 meshes to the linear inch and then suspended in normal saline.

RESULTS

Infection of Experimental and Resistant Mice (Experiment 1)

Of a batch of 120 mice, 60 were selected at random and each was given an oral dose of 5000 larvae. After an interval of 30 days all 120 were given a further dose of 5000 larvae. At various intervals of time thereafter ranging from 12 hours to 7 days, six mice from each group were killed and the lungs, mesenteric lymph nodes and intestinal wall examined for the presence of larvae. Previous observations had shown that in the mouse nearly all the larvae penetrate the wall of the large intestine, and hardly any do so further up the alimentary tract. The large intestines alone were therefore taken for examination, their contents being carefully removed by repeated washing.

The larvae recovered are shown in Fig. 1. It will be seen that there was no difference in the numbers recovered from the intestinal wall of resistant and susceptible mice nor any appreciable difference in those recovered from the mesenteric nodes. Considerably fewer larvae were recovered however from the lungs of the resistant mice than from those of the susceptible mice. It would appear that in the resistant animal the larvae are not prevented from penetrating the intestinal wall, nor from reaching the mesenteric nodes. The conclusion that the larvae fail to reach the lungs would seem to suggest itself but the possibility cannot be entirely excluded either that they pass through the lungs or are very rapidly destroyed or eliminated.

Infection of Experimental aud Resistant Rabbits (Experiment 2)

Forty-two rabbits were divided at random into two groups. Each rabbit of one group received an oral dose of 5,000 *D. filaria,* and all 42 a further dose of 50,000 larvae 30 days later. On the 1st, 2nd, 3rd, 4th, 5th, 7th and 8th day after infection 3 rabbits from each group were killed and the numbers of larvae in their lungs and lymph nodes estimated. The mean numbers recovered are indicated in Fig. 2. The results correspond well with those of experiment 1. There was little difference between the numbers of larvae recovered from the mesenteric nodes of resistant and susceptible animals but

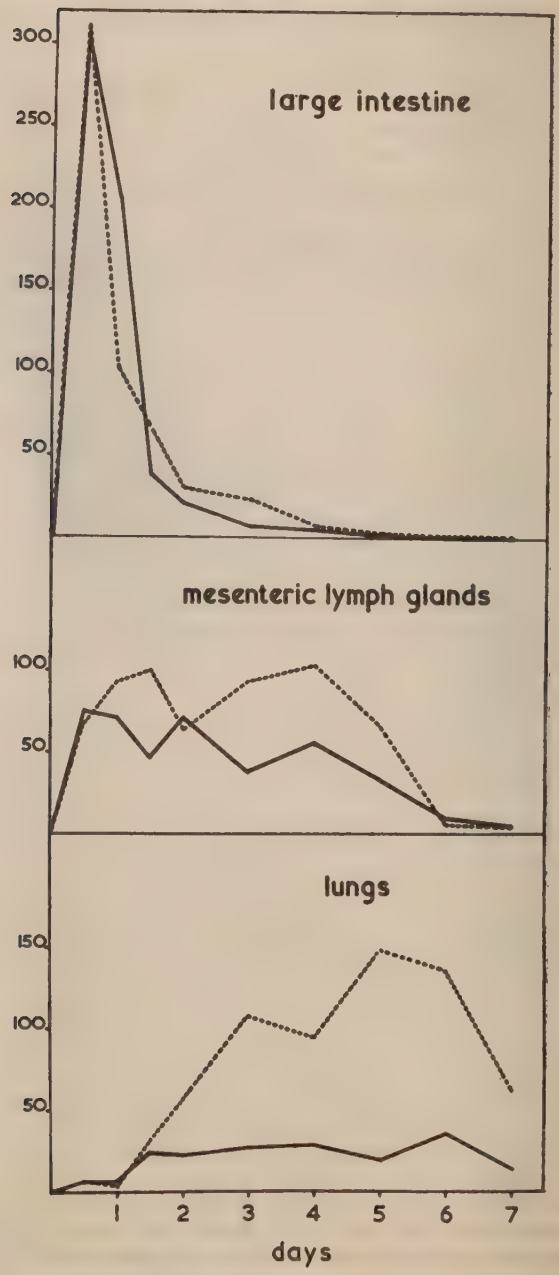


Fig. 1. Mean numbers of larvae recovered from mice in experiment 1.
Dotted line—susceptible mice.
Solid line—resistant mice.

a marked difference between the numbers recovered from the lungs. The gradual rise in the number of larvae recoverable from the lungs of the resistant rabbits is of interest and suggests that the larvae are being prevented from reaching the lungs.

Fig. 2

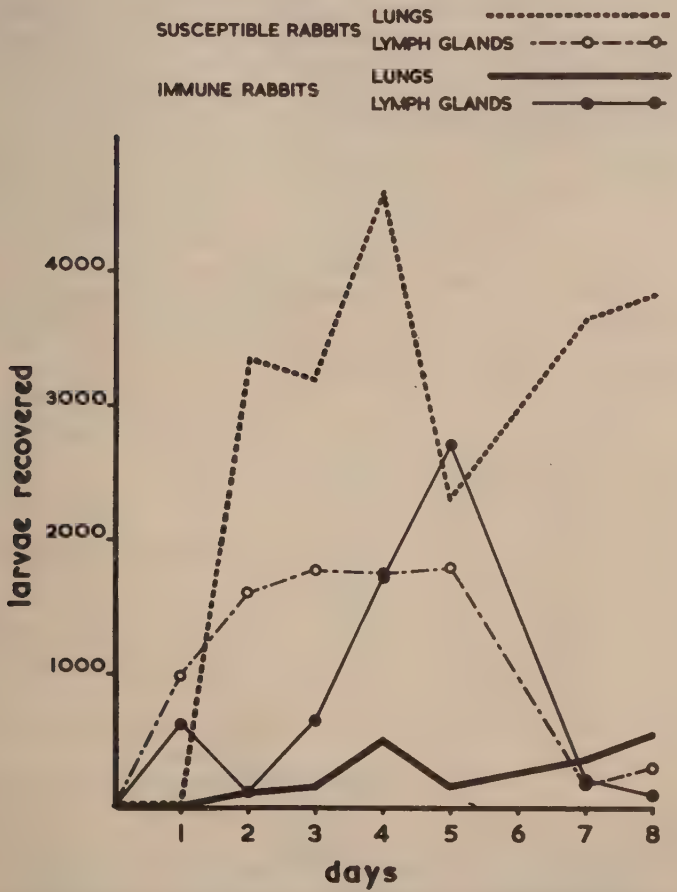


Fig. 2. Mean numbers of larvae recovered from lungs and mesenteric lymph glands of rabbits.

Infection of Experimental and Resistant Lambs (Experiment 3)

Ten lambs were divided at random into pairs and one of each pair was given an oral dose of larvae which represented 80 larvae per lb. body weight. After 113 days all ten received a dose of 8,000 larvae. Pairs were killed on the 5th, 6th, 7th, 8th and 9th

days and the lungs and mesenteric nodes finely minced and subjected to Baermann extraction in saline at 37°C. The numbers of larvae recovered are indicated in Fig. 3. In view of the small numbers of animals available too much regard should not be paid to the actual numbers recovered but the trend of events is clearly seen. The numbers of larvae in the lungs of the susceptible animal rise more steeply than in the resistant animal. Moreover, this rise coincides with a decrease in the numbers of larvae in the lymph nodes, a circumstance which suggests that the larvae do not first become established in the lungs of the resistant animals and are subsequently destroyed or eliminated.

Fig. 3

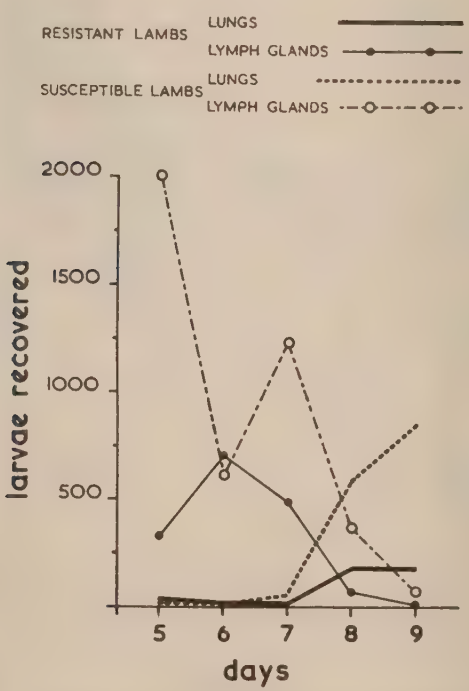


Fig. 3. Number of larvae recovered from sheep in experiment 3.

Experiment 4

The foregoing results suggest that though the larvae reach the mesenteric nodes of a resistant animal they fail to become established in the lungs, and they do not appear to be destroyed there or rapidly eliminated. It was decided to pursue the matter by allowing larvae to by-pass the mesenteric nodes by giving them intravenously.

Preliminary observations with sheep having shown that it is possible to establish infections of *D. filaria* by the intravenous administration of exsheathed third stage larvae, 2,500 larvae were administered intravenously to two groups each of 4 resistant and to two groups of susceptible mice. One group of resistant and one of susceptible mice were killed after 1 hour and the remainder after 24 hours. The lungs were finely chopped and subjected to Baermann extraction to give an estimate of the number of live larvae present. At the same time, two groups of resistant mice of the same batch, and two groups of susceptible controls were orally infected to confirm that these resistant mice did indeed show the same resistance as those in earlier experiments. The results are given in Table 1. It will be seen that when the mesenteric nodes were by-passed, equal numbers of larvae were recovered from the lungs of resistant and susceptible mice.

TABLE 1

MEAN NUMBERS OF LARVAE RECOVERED FROM MICE IN EXPERIMENT 4

	Time after infection	*Immune*	*Susceptible*
Lungs of intravenously infected mice	1 hour	71	79·25
	24 hours	68·75	44
Lungs of orally infected mice ..	2 days	3	7·5
	3 days	8·5	36·25
Lymph glands of orally infected mice	2 days	36·25	39·75
	3 days	21·25	34·25

The Microscopic Examination of Sections

Sections of the lungs of resistant and susceptible rabbits suggest that the larvae are not destroyed in the lungs of the resistant rabbit. The numbers of larvae seen reflected those recovered in Experiment 2 and there were no signs of disintegration or destruction of larvae. In the mesenteric nodes of rabbits there were signs of the destruction of some of the larvae and many were the centre of a tissue reaction, but this was seen equally in susceptible and resistant animals. In sections of the lymph nodes of mice, though only very few larvae appeared to be associated with any tissue reaction, this occurred in both susceptible and resistant mice. The numbers of larvae seen closely corresponded with those recovered in Experiment 2.

DISCUSSION

The interpretation of the results obtained in these experiments calls for considerable caution. Almost any procedure adopted in the study of the present problem must be akin to lifting an occasional paving stone and counting the ants on it in order to determine in what manner ants are moving about the pavement. This difficulty applies both to assessments of the number of worms present in an organ at different times and the microscopic examination of sections for, though it may be possible to deduce the occurrence of a process from such data, it is not easy to obtain any indication of its rate.

Although there is evidence concerning the possibilities outlined in the introduction to the present paper, it is not justifiable to draw more than tentative conclusions. It would appear that the larvae succeed in penetrating the intestinal wall of a resistant animal and are not destroyed there. Again, it would seem that such larvae as reach the lungs of a resistant animal are filtered out of the blood in the smaller vessels but are neither destroyed in, nor eliminated from, the lungs. If neither the lungs nor the large intestine appear to be the primary site of the protection mechanism, then it would follow that since they do not seem to reach the lungs, the larvae must be destroyed or lost somewhere between their entry into the mesenteric nodes and their passage through the thoracic duct.

Subsequent observations which are as yet incomplete, have suggested that in resistant sheep there may be an additional or alternative mechanism preventing the establishment of larvae that have reached the venous blood.

CONCLUSIONS

A series of experiments with *D. filaria* in sheep and small laboratory animals is described, designed to investigate the site of the mechanism by which worms are prevented from establishing themselves in the lungs of a resistant animal.

Neither the intestinal wall nor the lungs appears to be the primary site of this mechanism and the evidence suggests that in the resistant host the larvae are destroyed or lost in the mesenteric nodes or the lymphatic vessels.

REFERENCES

Hobmaier, A., and Hobmaier, M. (1929). *Munch. tierärztl. Wschr.*, **80**, 621.
Michel, J. F. (1956). *J. comp. Path.*, **66**, 241.

[*Received for publication, March 8th,* 1956]

DATA ON SHEEP REPRODUCTION DERIVED FROM ABATTOIR MATERIAL

By

G. H. Arthur

Royal Veterinary College, Streatley

INTRODUCTION

During the period January to March in each of the years 1951 to 1953, genitalia of sheep slaughtered at local abattoirs were collected and notes made on several aspects of reproductive function. The uteri were obtained mainly from crossbred Hampshire sheep which were approximately one year old at the time of slaughter.

Data were obtained on the size of the ovaries, the position and size of ovarian follicles and corpora lutea, the position of the foetuses in the uterus and their size, the disposition of the foetal membranes, the quantities of amniotic and allantoic fluid and the number of cotyledons, the position and size of the foetal testicles and the distribution of melanin.

MATERIALS

Seventy-nine specimens, comprising 24 non-pregnant uteri, 40 uteri pregnant with single foetuses, 13 uteri with twins and two

TABLE 1

OVINE REPRODUCTION (JANUARY - MARCH)

Ovulation Frequency

		Left ovary	*Right ovary*
24	Non-pregnant uteri	15	16
40	Pregnant uteri with singles	20	27
13	„ „ „ twins	15	11
2	„ „ „ triplets	3	2
79	Overall incidence	53	56

Migration of Embryos

	Left to right	*Right to left*
Single foetuses	2	0
Twin „	1	3
Triplet „	0	0

Loss of Eggs or Embryos

	From left ovary	*From right ovary*
Single foetuses	5	2
Twin „	0	0
Triplet „	0	0

Overall percentage loss of embryos=8·9.

Cornual Distribution of Foetuses

	Left Cornu	*Right Cornu*
Single pregnancies	17	23
Twin „	13	13
Triplet „	3	3
Total	33	39

TABLE 2

OVINE REPRODUCTION: SINGLE PREGNANANCIES

Corpora lutea		*Foetuses*			*Amniotic fluid*		*Allantoic fluid*		*Total fluids* (*ml.*)	*Foetal body length* (*cm.*)	*Position of testes*		
Left Ovary	*Right Ovary*	*Left Cornua*	*Right Cornua*	*Presentation*	*Left*	*Right*	*Left*	*Right*			*Abdomen*	*Ing. Canal*	*Scrotum*
	1		F	A		6		40	46	2·8			
	1	?								3·0			
1		M								3·0	+		
	1		F			10		15	25	3·4			
1		F			14		40		54	4·4			
1		M		A	FLUIDS LOST					5·0	+		
1		F			95		40		135	8·75			
1		F		A	72		75		147	10·0			
	1+1		M	A		160		80	240	10·0	+		
1		F		A	180		70		250	11·25			
	1		F	P		150		75	225	12·0			
1		F			25		15		40	12·5			
1 + 1		F		A			65			15·0			
	1		M	A		200		50	250	15·0	+		
	1	F		P	450		5		455	17·5			
1 + 1		M		A	425		110		535	17·5			
	1		M	A	FLUIDS LOST					18·75			
	1		F	A		350		65	415	19·5			
	1		M	A		415		130	545	20·0		+	
1	1		F	P		550		105	655	22·5			

Corpora lutea		*Foetuses*			*Amniotic fluid*		*Allantoic fluid*		*Total fluids (ml.)*	*Foetal body length (cm.)*	*Position of testes*		
Left Ovary	*Right Ovary*	*Left Cornua*	*Right Cornua*	*Presentation*	*Left*	*Right*	*Left*	*Right*			*Abdomen*	*Ing. Canal*	*Scrotum*
1		F		A	565		100		665	23·7			
	1		F	P					655	23·7			
	1		M	P		460		650	1110	25			+
	1		F	A		825		160	985	25			
1		M		A	280		1235		1515	27			
1		F		A	150		660		810	27			
	1		M	A		530		110	640	27·5			
	1		M	P		500		90	590	27·5		+	+
	1		F	A					570	28·7			
	1		M	A		825		170	995	29			+
1		M		P	700		85		785	31·2			+
1	1		F	P		900		1425	2325	32			
	1		F	A		690		40	730	32·5			
	1+1		F	A	FLUIDS LOST					32·5			
1	1		M	A		110		450	560	35			+
	1		M	A					650	35			
	1		F	A		480		75	555	37·5			
1		F		A	150		660		810	45			
1		M		A	280		1235		1515	45			+

KEY: M=Male. F=Female. A=Anterior. P=Posterior. T=Transverse. X=Triplets.

with triplets, were examined. The data were then assembled and arranged in Tables 1 to 4 which form the essence of this report. In Table 1 collective data on the functional activity of the left and right ovaries and uterine cornua are shown. In this work the presence of a functional corpus luteum in an ovary was taken as evidence of ovulation from that ovary. From the ovulation frequency and foetal distribution the extent of embryonic migration and death of embryos (including loss of eggs) was calculated.

Table 2 shows the details of ovulation frequency, cornual distribution, sex and size of foetuses, the presentation of the foetus, the quantities of foetal fluids and the position of the foetal testes in uteri pregnant with singles. In Table 3 the data for multiple pregnancies are arranged under the same headings. Table 4 summarises the data on foetal presentation.

RESULTS

Ovulation Frequency

When the data from all specimens showing corpora lutea are considered it will be seen that both ovaries functioned similarly, the ratio for the 79 specimens being 56 ovulations from the right ovary to 53 from the left.

The evidence from the ovaries of the 24 non-pregnant specimens showed that in the months January to March, 18 of the sheep had been slaughtered in dioestrus, two in or very near oestrus and four in anoestrus. It is possible, however, that two of the anoestrus cases had been pregnant and that resorption of a foetus had recently occurred in each case. The interesting inference from these observations is that British sheep, when well-fed, may have breeding cycles at this time of year when the length of daylight is on the increase.

Foetal Distribution

In the aggregate of single, twin and triplet pregnancies, 33 foetuses were found in the left uterine horn and 39 in the right. This slight preponderance of the right side recalls similar trends obtained from both the mare and dairy cow. Factors which obviously have a bearing on the relationship between ovulation frequency and cornual distribution are the phenomena of intercornual migration of embryos and egg-embryonic death. In fact, in this study embryonic migration had occurred on three occasions in each direction; an illustration of left to right migration is in Fig. 2 which shows two corpora lutea in the left ovary (none in the right) and a foetus in the right uterine horn.

There were seven instances of lost ova or embryos; all occurred in the single pregnancy specimens, (Figs. 1 and 2). This gave a percentage egg-embryo mortality of 8·9. Winters and Feuffel (1936) using the same criteria, as in the present study, on 46 pregnant ewe specimens at Minnesota, recorded that 8 per cent of ovine eggs did not develop. In two of the observations recorded in Table 2

TABLE 3

OVINE REPRODUCTION: MULTIPLE PREGNANCIES

Corpora lutea		*Foetuses*			*Amniotic fluid*		*Allantoic fluid*		*Total fluids (ml.)*	*Foetal body length (cm.)*	*Position of testes*		
Left Ovary	*Right Ovary*	*Left Cornua*	*Right Cornua*	*Presentation*	*Left*	*Right*	*Left*	*Right*			*Abdomen*	*Ing. Canal*	*Scrotum*
1+1		1	1	P : A						1·2			
1X	1	1	2							1·8			
1	1	1	1							2·2			
1	1	F	F	P : P	135	135	45		315	10			
1	1	F	M	A : P					430	11·25	+		
1	1	M	M	T : P	115	80	50	50	295	11·9	+		
1+1		M	F	A : A					700	14	+		
1	1	F	M	P : P	375	565	325	460	1725	22·5		+	+
	1+1	F	M	A : P	290	525	40	180	1035	22·5			+
1	1	F	F	P : P	605	520	160	65	1350	26·5			
1	1	F	F	A : P	675	660	220	375	1930	27			
1	1	M	M	A : A	680	950	265	200	2095	30			
1+1		M	M	A : A	770	690	1210	770	3440	30			+
1	1	M	F	A : A	FLUIDS LOST					35			+
1+1X	1	F : M	M	A : A : A	FLUIDS LOST					42·5			+

KEY: M = Male. F = Female. A = Anterior. P = Posterior. T = Transverse. X = Triplets.

where there were two corpora lutea but only one developing foetus, there was a disintegrating chorion attached to the normal chorion. In a study of this kind in which macroscopic features only were ascertained it is obviously much more likely—on account of persistence of the corpora lutea—that evidence of embryonic death would be apparent in the definitely pregnant uteri than in those which appeared empty. It is not impossible, however, that a proportion of the uteri which were classified as non-pregnant might have suffered embryonic loss. There seems little doubt that this is an aspect of ovine fertility or fecundity which is of some importance.

Monozygous Twinning

In equating the number of corpora lutea present with the number of foetuses, one of the triplet pregnancies was of interest. There was one normal C.L. in each ovary, but in the uterus there were two embryos in the right horn and one in the left. This was a pregnancy of about one month duration and there was no sign of another regressing C.L. The two foetuses in the right horn shared a common chorion which was separate from that of the foetus in the left horn. The conclusion, therefore, was that an egg released from the right ovary had given rise to monozygous twins in the right cornu. An exactly similar state of affairs was present in another set of triplets, seen outside this series. Here the FBL was 3·5 cm. No record can be found in the literature of identical twinning in sheep, but in view of the not infrequent occurrence of double monsters in this species, it would be surprising if it did not occur. In the other case of triplets where the mother had been killed near term, two foetuses in the left horn and one in the right corresponded to 2 C.L. in the left ovary and one in the right. As was to be expected at this late stage of gestation, one chorion was common to all three foetuses.

The Position and Mobility of the Foetuses

The incidence of anterior, posterior and transverse presentation of the foetus *in utero* is shown in Table 4. Of 60 foetuses in which the polarity of the foetuses was noted, 40 were presented anteriorly,

TABLE 4

INCIDENCE OF FOETAL PRESENTATIONS

	Anterior	*Posterior*	*Transverse*
Single Foetuses	25	8	—
Twin „	12	11	1
Triplet „	3	—	—
Total	40	19	1

In some early pregnancies presentation was not determined.

19 posteriorly and one transversely. The difference in presentation according to whether single or twin foetuses were carried was rather striking, although, admittedly, the number of observations made was small. Thus, for single pregnancies there were about three anterior to every one posterior presentation (25:8) whereas in the case of twins the numbers were almost equal (12:11). No reason was found for the preponderance of anterior presentation; the ratio of 3:1 for single pregnancy is very different from the 19:1 anterior to posterior quoted by Williams (1931) for the dairy cow and is more in line with data obtained from the sow and bitch. It must be pointed out that the figures here recorded may not be representative of the final presentations at birth, on which no figures are available. The same criticism may be applied to the work of Curson and Quinlan (1934). They examined 33 sheep uteri containing single foetuses of up to three months and found 20 cranial and 13 caudal presentations; in four twin pregnancies these workers found 4 anterior and 4 posterior presentations.

No attempt was made in the present study to determine the position of the foetus about its longitudinal axis as it was believed that this might change after death. The same might be said of posture; in this connection, however, the vast majority of the foetuses were in the position of "universal flexion," although it was interesting to find that two of the 72 single foetuses in anterior presentation showed the head deviated laterally (Fig. 7). Curson and Quinlan (1934) found one foetus, a twin, with lateral deviation out of a total of 41 examined.

Before leaving the subject of foetal orientation, it should be stated that the ovine foetus seems to have complete mobility around both longitudinal and transverse axes within the amnion at least up to 3½ months of gestation (Figs. 3 and 8). It would seem, therefore, that provided foetal movement is sufficiently vigorous, change of presentation may occasionally occur up to this stage of gestation. That such rotation did occur around a transverse axis, was shown in a number of cases by varying degrees of torsion of the amniotic portion of the umbilical cord. The greatest torsion observed was 2½ revolutions and this had apparently produced no ill effect on gestation, although it is conceivable that violent foetal mobility might be stimulated just before death. This approaches the condition of the porcine foetus in which torsion of the amniotic cord of up to 4 times has been seen.

The Foetal Membranes

In sheep the chorion is extensive and even in single pregnancies tends to occupy most of the non-gravid cornu. Quite frequently it was not vascularised to its extremity by the allantois, so that an extensive "necrotic tip" was often present, (Fig. 3). Most of the allantoic fluid occupied the two poles of the allantois, for there is only a narrow channel of this organ running over the foetal area

and connecting the extremities which lie one in each horn (Fig. 4). The average number of cotyledons was 74, in four rows.

Where twin foetuses were present it was usual for the chorions to fuse at their point of contact, but the allantoic vesicles remained separate. A point of considerable interest was whether the allantoic vessels of adjacent foetuses anastomosed. Nothing like the clear anastomoses of cattle vessels was seen, but the impression gained was that anastomosis of small vessels did occur occasionally and allantoic vessels from separate foetuses have been seen running to the same cotyledon. In only one case of unlike sexed twins, however, was there a suggestion that the female was abnormal; in this case the clitoris was large and projected from the vulva, but the internal genital organs appeared normal. Even in a case of triplets, one female and two males, where the ewe died near term, the ewe lamb had normal genitalia. It is evident, therefore, that no clear-cut case of an ovine freemartin has yet been described.

The total quantity of foetal fluid increased progressively throughout gestation with advancing age of the foetus, and the data confirmed work already done by Malan and Curson (1937) and by Cloete (1939) on Merino sheep. The behaviour of the separate allantoic and amniotic fluids is compared with the foetal fluids of cattle in another paper (Arthur, 1956).

The Foetal Gonads

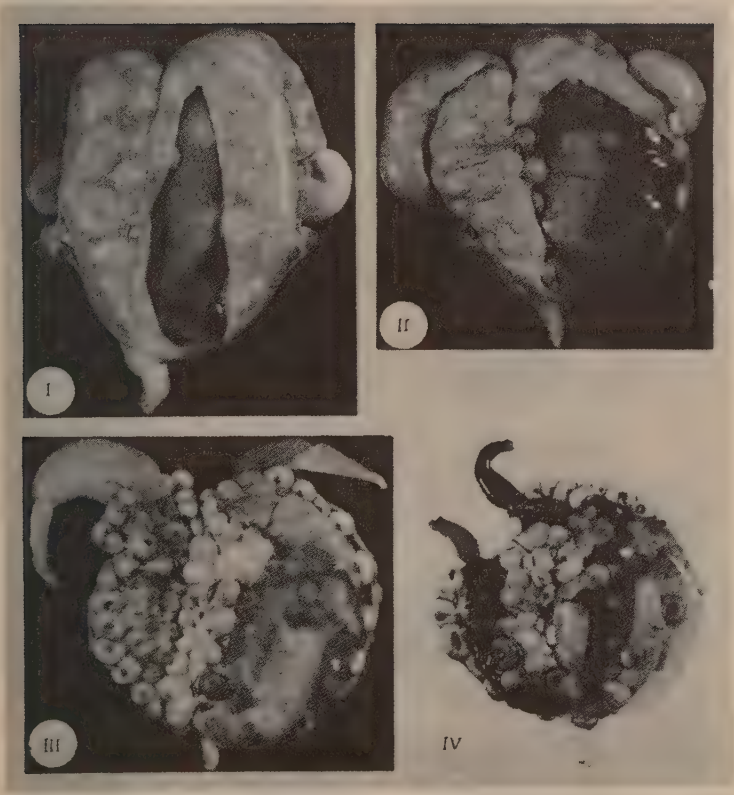
The ovaries and testes of the foetal sheep show a gradual growth pattern throughout gestation and nothing was seen to compare with phenomena observed in the horse where a massive proliferation of interstitial tissue causes relatively huge foetal gonads at one stage of gestation. A striking feature, however, was the early period in gestation at which the testes passed from the abdomen to the scrotum. This subject is discussed elsewhere (Arthur, 1956).

Further Observations

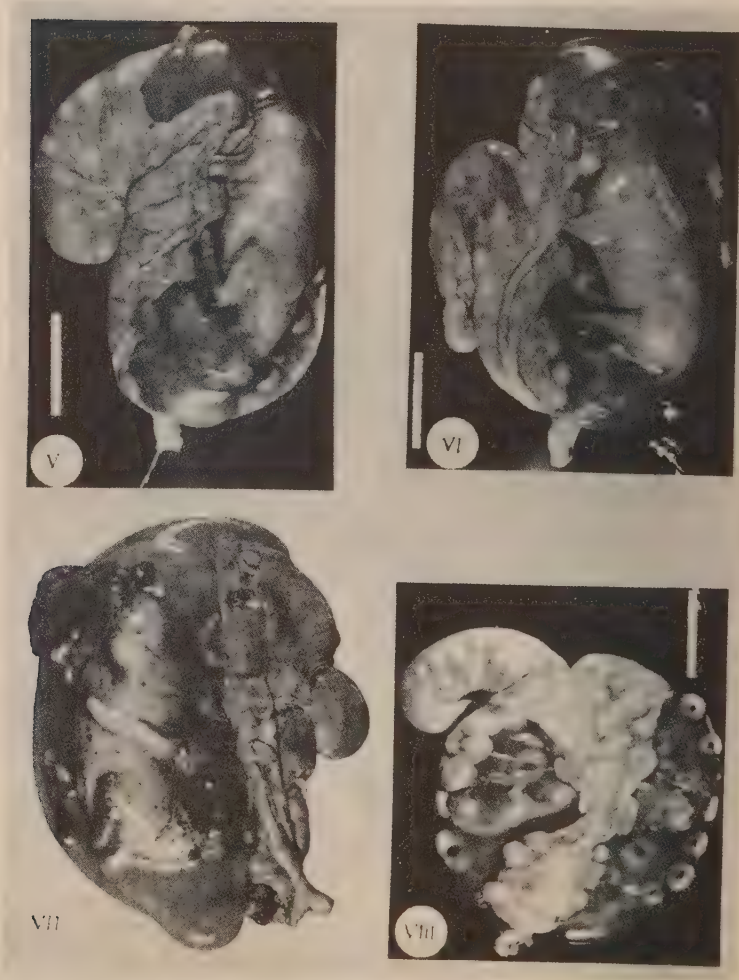
A mucus seal in the cervix was present in all pregnant specimens, but was not as conspicuous as in the cow when viewed from the vulva because the cervix of the ewe is contained within a kind of canopy which projects back into the vagina and is entered by a vertical slit from the vagina.

Melanin deposition was a feature in the endometrium of the non-pregnant uteri. It was usually intercotyledonary, but occasionally only the caruncles were coloured. It was noteworthy that in none of the pregnant uteri examined was any melanin observed in the uterus.

Cystic corpora lutea were observed, but no case of ovaro-bursal adhesion was encountered. The specimens were free from developmental abnormalities and no macroscopic evidence of infection was seen.



To face page 352


V
VI
VII
VIII

CONCLUSIONS

The ovaries of the non-pregnant ewe show cyclic activity until March. Both function equally.

There is a slight preponderance of pregnancies in the right uterine horn. Occasionally an embryo migrates to the other horn. Loss of eggs and embryos may amount to 8·9 per cent.

Monozygous twinning may occur but the occurrence of ovine freemartins is unlikely.

Three out of four single foetuses present anteriorly but half the twins are in posterior presentation.

ACKNOWLEDGMENTS

I wish to record my thanks to Messrs. W. L. King and P. Henfrey for technical assistance.

REFERENCES

Arthur, G. H. (1956). *In press.*
Cloete, J. H. L. (1939). *Onderstepoort J. vet. Sci.*, **13**, 418.
Curson, H. H., and Quinlan, J. B. (1934). *Ibid.*, **2**, 567.
Malan, A. P., and Curson, H. H. (1937). *Ibid.*, **8**, 417.
Williams W. L. (1931). *Veterinary Obstetrics,* 2nd Ed., W. L. Williams; New York.
Winters, L. M., and Feuffel, G. (1936). *Univ. Minnesota agri. Exp. Sta. Tech. Bull.* No. 118.

[*Received for publication, March 8th,* 1956]

Fig. 1. Egg-embryonic loss, from left side. Corpus luteum in each ovary; one foetus in right horn.

Fig. 2. Embryonic Migration and Loss. Two C.L. in left ovary; one foetus in right horn.

Fig. 3. Foetal Mobility. Same specimen as in Fig. 2. Presentation has been artificially changed from anterior to posterior (FBL 20 cm.) Also shows placentation in non-gravid left horn and large necrotic tips at extremities of chorion.

Fig. 4. Placental Topography. Same specimen as in Figs. 2 and 3; allantois injected to show relative volume and disposition of allantois and amnion.

Fig. 5. Foetal Presentation. Impossible, after death, to alter this anterior presentation of a 27·5 cm. foetus.

Fig. 6. Impossible, after death, to alter this posterior presentation of a 32 cm. foetus.

Fig. 7. Foetal Presentation. Foetus in anterior presentation in left horn, with lateral deviation of its head.

Fig. 8. Foetal Presentation. Twin pregnancy. Left foetus in transverse presentation, right foetus was posterior. At this 11·9 cm. stage, post-mortem, the presentation can be altered easily.

HAEMATOLOGY OF EXPERIMENTAL BRACKEN POISONING OF CATTLE

I. Changes in Blood and Bone Marrow

II. Attempts to Modify the Course of the Bone Marrow Damage

By

J. M. Naftalin* and G. H. Cushnie

Rowett Research Institute, Bucksburn, Aberdeenshire

INTRODUCTION

We have previously shown that the essential lesion in cattle and calves fed experimentally on bracken fern (*Pteris aquilina*) was bone marrow damage (Naftalin and Cushnie, 1954 a, b). We put forward the alternative hypotheses that the bone marrow damage was caused by a poison in the bracken itself or arising from microbial attack in the rumen, or that it was due to the lack of some essential nutrient.

We now describe changes in blood and bone marrow of calves feeding on bracken under experimental conditions and tests of the above hypotheses.

EXPERIMENTAL PROCEDURES

Animals. The experiments were done with one female and 25 male Ayrshire calves between 3 and 5 months old. An eighteen month old heifer was used for one experiment. The heifer and some of the calves were fitted with permanent rumen or abomasal cannulae before the experiments began.

Bracken feeding. The animals were allowed to eat to appetite and, with the exception of two calves, the bracken was eaten without apparent distaste. In June-August, 1954, fresh green bracken was cut every alternate day for feeding to animals in Experiments 1b, 2b and c, 3 and 6. The bracken was kept indoors after cutting and fed without further chopping. Sun-dried bracken harvested early in July, 1952 was used for Experiments 4 and 5. The dried bracken was chopped in a hay cutter before feeding. Experiments 1a and 2a were done with sun-dried bracken harvested late in August, 1953. This bracken was more fibrous than the 1952 sample.

Steamed bracken. For Experiments 2 a, b and c the bracken was placed in open-meshed sacks and exposed to flowing steam for 1 hour in a dairy type steam sterilizer. The temperature in the middle of the sack of bracken was recorded by a thermocouple and reached 100°C within 5 minutes. Enough bracken for two days feeding was steamed at one time.

Dietary supplements (*Experiment* 6). Two calves were given the following supplements to their diet of fresh green bracken:— Calf 33a received 37 g. of casein hydrolysate** three times a day for 19 days and 50 g. for 14 days.

**Present address:* Veterinary Laboratory, P.O.B. 154, Haifa, Israel.

***'Pronutrin,' dried enzymic digest of lactic casein, Herts Pharmaceuticals Ltd., Welwyn Garden City, England.

Calf 40 received the following mixture 3 times daily:— dried skim milk, 64 g.; dried brewer's yeast, 8 g.; maize starch, 20 g.; McCollum's salt mixture 185+Fe, 40 g. All the supplements were given via the abomasal cannula in 300 ml. of water.

Administration of group B vitamins (*Experiment* 3). Calf 12 was given daily intramuscular injections of the following mixture of the group B vitamins:— aneurin 15 mg. riboflavin 30 mg. pyridoxin 15 mg.; Ca pantothenate 100 mg.; biotin 1 mg.; folic acid 15 mg.; inositol 100 mg.; nicotinic acid 200 mg.; B_{12} 100γ.

Calf 13 was given vitamin B_1 directly into the rumen via the cannula, receiving 720 mg. daily on Monday to Friday and 560 mg. on Saturday and Sunday.

Transfers of rumen liquor (*Experiments* 4 *and* 5). In Experiment 4, a heifer was fed dried green bracken and hay and some of the liquor was removed twice daily from the rumen. This liquor was strained through muslin and then poured into the rumen of Calf 7 on a normal diet of concentrates, swedes and hay. Calf 7 received 5 litres of liquor daily for 42 days. In Experiment 5, a heifer was fed normally on a diet of either turnips and hay or on fresh grass only. Normal rumen liquor was transferred from this animal to the rumens of 5 calves and 1 heifer while they were on a diet of fresh green or sun-dried green bracken.

The normal rumen liquor was transferred twice daily into the rumens of the bracken-fed animals (a) when the bone marrow was already damaged (3 calves and 1 heifer) and (b) from the beginning of the experiment (2 calves). The heifer received 10 to 14 litres daily while the calves received on average 4 to 10 litres daily.

Haematology. The haematological methods used were those previously described (Naftalin and Cushnie, 1954 a and b).

Bone marrow biopsy. Serial bone marrow aspirations were made from the sternum. The degree of cellularity in the marrow of cases of bracken poisoning was estimated by comparison with marrow from the sterna of normal animals. These serial biopsy specimens were also compared with large histological sections from the sterna of normal calves and with sections of sterna from calves killed on the 10th, 18th and 24th day after the beginning of an experiment.

RESULTS

The complete data obtained from all the experiments carried out in this work are too extensive for publication here and therefore only representative results and essential findings of each experiment will be included in this paper.

Calves Fed on Sun-dried Green Bracken (1953 sample) (Experiment 1a)

The purpose of this experiment was to study the serial haematological changes in blood and sternal bone marrow of 5 calves on a diet of sun-dried green bracken (Table 1). The bracken was not very palatable and the calves ate little. The average daily consumption was bracken: $2\frac{3}{4}$ lb.; hay: 1 lb. From the start of the

TABLE 1

RESULTS OF FEEDING CALVES ON DRIED BRACKEN (AUGUST 1953 SAMPLE)

Calf No. and sex	*Days lived after eating bracken*	*Weight (lb.) during experiment*		*Remarks*
		Beginning	*End*	
26 ♂	27	137	112	Steady loss of weight (0·9 lb./day). Terminal syndrome not haemorrhagic. Temp. 98·0°F. Heart rate 46 beats/min.
27 ♂	50	271	203	Steady loss of weight (1·3 lb./day). Died without signs of bracken poisoning. Temp. 99·0°F. Heart rate 40 beats/min.
28 ♂	61	241	184	Steady loss of weight (0·7 – 0·9 lb./day). Died without signs of bracken poisoning. Temp. 97·0°F. Heart rate 35 beats/min.
29 ♂	37	208	141	Steady loss of weight (1·8 lb./day). Died without signs of bracken poisoning. Temp. 97·0°F. Heart rate 33 beats/min.
31 ♂	26	154	119	Steady loss of weight (1·3 lb./day). Died without signs of bracken poisoning. Temp. 96°F. Heart rate 40 beats/min.

experiment, the animals lost weight and condition. As the experiment progressed, the rectal temperature and heart rate of each animal became sub-normal.

Bone marrow. The bone marrow of all five calves showed a decreased cellularity between the 19th and 27th day. In Calf 26 (Table 2) there was no relative decrease in the intermediate and late normoblasts, but the cells of the erythroid, myeloid and megakaryocytes series were reduced in numbers. The bone marrow of two other calves presented a similar picture until a few days before death, when the red cell precursors were relatively reduced. In the remaining two calves, the sternal bone marrow picture was different from Calf 26 in that there was a relative reduction in red cell precursors as early as 12 and 14 days after feeding on dried bracken.

On post-mortem examination, the sternal bone marrow of Calf 26 and one other still contained many cells (Fig. 17); the bone marrows of the other three calves were on the whole less cellular than those of Calf 26. However, in all these calves, even at the end of the experiment, differential counts of the sternal bone marrow showed cells at all stages of maturation from blasts to mature cells. There was little or no oedema of the stroma; the capillaries were dilated in some areas.

Peripheral blood. The number of red cells per c.mm., Hb/g. per cent, and packed cell volume showed steady increases. The white cells of Calf 26 increased from 9,000 to 12,000 per c.mm. in 21 days, while in the other four calves there was only a very slight fall from the initial values. In all calves the platelets were reduced in number, the lowest value reached being 104,000 per c.mm. in one of the calves in 24 days.

Calves Fed on Fresh Green Bracken (1954 sample) (Experiment 1b)

The purpose of this experiment was to compare the effect of feeding fresh green bracken with the effect of the dried bracken sample in Experiment 1a. Seven calves were used in a study of the serial changes in blood and bone marrow. In general, animals fed fresh green bracken remained well during the first 1 to 3 weeks of the experiment but lost condition thereafter. In the first week they lost a few pounds in weight and either maintained this weight or quickly regained their original weight. The exception was Calf 45 which ate an average of only 3½ lb. of bracken a day and died without signs of bracken poisoning on the 7th day of the experiment having lost 38 lb.

As previously reported (Naftalin and Cushnie, 1954 b) bracken poisoning was accompanied by a slowing of the heart rate, although the rate was increased shortly before death. There was also a terminal rise in temperature from 105 to 107°F. Some of the general results of this experiment are summarized in Table 3.

Bone marrow. Changes in the sternal bone marrow of the calves

TABLE 2

SERIAL DIFFERENTIAL COUNTS PER CENT OF STERNAL BONE MARROW

Calf 26 fed on dried bracken (August 1953 sample)

Date	*16th* Oct.*	*26th Oct.*	*2nd Nov.*	*6th Nov.*	*10th Nov.*	*Remarks on smears and histological sections*
Haemocytoblast	1·4	1·2	1·6	0·8	0·8	The sections appeared to be of normal cellularity till 10th November (24th day) when a slight decrease was observed, and the capillaries appeared dilated.
Normoblast:						
Early	4·8	2·8	4·0	3·0	2·0	
Intermediate	24·0	22·0	13·0	20·0	21·0	
Late	28·2	27·2	14·6	14·0	18·6	The calf died 3 days later and *post-mortem* sections of the sternum showed that the lesions were patchy; some areas are of apparently normal cellularity, while at the other extreme there are areas devoid of haematopoietic tissue with slight degree of oedema of the stroma, dilated capillaries and small haemorrhages.
Blasts	2·2	1·6	4·2	3·2	1·6	
Pro-myelocyte	0·4	1·4	1·2	2·6	0·8	
Myelocyte (Neutrophilic)	0·2	2·0	1·2	2·8	1·6	
Metamyelocyte (Neutrophilic)	6·8	19·2	11·0	15·0	11·8	
Polymorph (Neutrophilic)	24·2	14·2	28·0	20·0	20·0	
Myelocyte (Eosinophilic)	1·2	0·0	0·8	0·0	0·2	
Metamyelocyte (Eosinophilic)	3·8	3·2	3·0	2·8	2·2	
Eosinophil (Eosinophilic)	0·2	0·0	1·2	0·4	0·4	
Basophil	0·0	0·0	0·0	0·2	0·0	
Lymphocytes	1·2	4·4	8·6	13·6	16·8	
Monocytes	0·4	0·4	7·6	0·8	1·0	
Plasma cells	0·0	0·0	0·0	0·0	0·0	
Megakaryocytes	0·0	0·0	0·0	0·6	0·0	
Reticulum cells	0·0	0·4	0·0	0·2	1·2	

*Normal Count.

were observed 10 to 15 days after the start of the experiment. These early changes were (1) diminution in the degree of cellularity affecting myeloid and erythroid tissue and (2) a severe relative (percentage) reduction of the intermediate and late normoblasts (Figs. 1 and 2, Table 4). The decrease in cellularity continued during the next few weeks, affecting the most primitive cells of the erythroid, myeloid and megakaryocyte series. In the later stages of poisoning there was a preponderance of polymorphs in the marrow; these in turn disappeared. By the 22nd to 27th day, the sternal bone-marrows of 4 of the calves in this experiment contained only scanty groups of haematopoietic tissue (Figs. 3 and 4).

Large histological sections of several parts of the sterna of calves killed on the 10th, 18th and 24th day after the start of the experiment confirmed the findings of the biopsies. Even after 10 days, a diminished cellularity was evident in the bone marrow; the predominating cells were metamyelocytes and polymorphs. Sections from the sterna of animals in the terminal phase of bracken poisoning showed that the marrow was devoid or almost devoid of haematopoietic cells; in most sections reticulum cells and endothelial cells of the capillaries persisted in an oedematous or haemorrhagic stroma (Figs. 5 and 6). The capillaries were often dilated: occasional fat cells could also be seen. However, in some sections the marrow consisted merely of a loose stroma devoid of cells.

A phenomenon worthy of note was observed in the bone marrow of two calves in this experiment. In Calf 14 (Table 4) after thirty-one days the cellularity of the marrow was very poor and continued so until the 41st day when scanty groups of early and intermediate normoblasts appeared in the marrow flecks (Figs. 14-16 and 18-20). By the 45th day these cells had increased in number and the clumps contained mainly erythroid precursors. Their numbers were sufficient to influence greatly the differential (percentage) count of the marrow because the cells of the myeloid series were so scanty. However, there did not appear to be a true regeneration and by the 48th day these "clumps" had disappeared from the smears. The calf died on the 49th day. Blast cells were relatively increased in the marrow on the 41st day but their numbers were insufficient to modify the marrow picture observed in the biopsies of the 45th and 48th day.

This temporary reappearance of primitive cells when bone marrow damage was well advanced was also observed in 4 of the animals in Experiment 5.

Peripheral blood. In general, the results obtained in Experiment 1b correspond with those previously published (Naftalin and Cushnie, 1954 a and b). The number of red cells and Hb/g. per cent in the venous blood showed little change throughout the experiments unless severe bleeding took place in the terminal stages. Platelets and leucocytes were always greatly reduced in numbers as the

TABLE 3

RESULTS OF FEEDING CALVES ON FRESH GREEN BRACKEN (1954 SAMPLES)

Calf No. and sex	*Day killed (K) or died (D) after eating bracken*	*Weight (lb.) during experiment*		*Remarks*
		Beginning	*End*	
45 ♂	7(D)	145	107	Ate little bracken. Average 3½ lb. daily. Died without signs of bracken poisoning; bone marrow not normal.
32A ♂	10(K)	179	159	Ate average of 10 lb. fresh green bracken daily.
43 ♂	18(K)	135	120	Ate average of 8½ lb. fresh green bracken daily.
41 ♂	24(K)	199	194	Ate average of 17½ lb. fresh green bracken daily.
46 ♂	30(D)	162	137	Ate average of 10½ lb. fresh green bracken daily.

Sections made from sterna obtained post-mortem confirmed the findings of serial biopsies. Bone marrow damaged after 10 days; reduction in cellularity progressing to aplasia. Megakaryocytes with bizarre nuclei appeared after 10 days but in this acute experiment megakaryocytes many with 3–6 nuclei persist till the 30th day.

disease progressed. It is of interest to note that despite an increase of blast cells in the marrow of Calf 14 (Table 4) on the 41st day, the white cells and platelets in peripheral blood continued to decrease in numbers.

Feeding with "Steamed" Bracken (Experiment 2 a, b and c.)

The purpose of these experiments was to study the effects of "steaming" bracken in the fresh and dried state at 100°C for 1 hour on the course of the disease and on the haematological changes. General results of these experiments are summarized in Table 5.

"Steamed" Dried Bracken (August 1953 sample) (Calf 32).

The course of this experiment followed that described for calves in Experiment 1a which were fed the same sample of dried bracken unsteamed at the same time. Calf 32 lost weight and condition from the beginning of the experiment. The changes in the peripheral blood counts, sternal bone marrow biopsies and post-mortem bone marrow sections were also similar to those described for Calf 26 (Experiment 1a, Table 2).

"Steamed" Fresh Green Bracken, (June-July, 1954 sample) (Calf 48)

The course of the disease in this animal was similar to that of a calf which was fed as a control on fresh unsteamed bracken at the same time (June to July 1954). Calf 48 was killed when moribund late on the 30th day and the control died early on the 30th day.

No essential differences in post mortem appearances were noted but Calf 48 had a little subcutaneous, omental, pericardial and perirenal fat; in the control calf fat was absent in these regions.

Bone marrow and peripheral blood. Bone marrow damage was similar in both Calf 48 (Table 6) and the control which was eating the same sample of bracken unsteamed. Damage was evident after 10 days on the bracken diet and the terminal bone marrow pictures were the same; the peripheral blood data of both calves were also similar. Both calves had signs typical of bracken poisoning.

"Steamed" Fresh Green Bracken, (July-August, 1954 sample) (Calf 35)

Bone marrow. In Calf 35 (Table 7) the sternal bone marrow showed decrease in cellularity on the 19th day affecting both erythroid and myeloid cells. By the 28th day the intermediate and late normoblasts showed a relatively greater decrease. On the 36th day the calf only ate 2 to 3 lb. "steamed" bracken but all the hay offered (1 lb.). Sternal bone marrow biopsy on this day showed a very damaged marrow containing only scanty cells. On the 39th day the marrow was further damaged. Three days later the marrow showed an increase in both primitive erythroid and myeloid cells. This was correlated with an increased cellularity (Figs. 21-23). Thereafter the early and late normoblasts increased relatively and in numbers, but the blast cells after reaching 6·4 per cent on the

TABLE 4

SERIAL DIFFERENTIAL COUNTS PER CENT OF STERNAL BONE MARROW

Calf 14 fed on fresh green bracken and hay.

Date	*4th* Aug.*	*14th Aug.*	*25th Aug.*	*4th Sept.*	*14th Sept.*	*18th Sept.*	*21st Sept.*	*Remarks on smears and histological sections*
Haemocytoblast	0·6	0·6	0·4	0·0	0·0	1·0	0·0	*4th August:* Normal degree of cellularity.
Normoblast:								
Early	2·2	1·2	0·6	0·2	1·0	5·0	0·5	*14th August:* Marrow appears less cellular than on 4th August. Greater relative decrease in intermediate and late normoblasts.
Intermediate	20·2	8·6	9·4	0·2	2·0	15·0	0·5	
Late	22·4	9·4	5·4	0·2	1·0	5·5	0·0	
Blasts	2·8	2·8	2·0	0·4	2·5	1·5	0·0	*25th August:* Poorly cellular marrow.
Pro-myelocyte	0·2	0·8	0·6	0·0	0·0	1·0	0·0	
Myelocyte (Neutrophilic)	1·8	2·0	2·0	0·0	0·0	0·5	0·0	*4th Sept.:* Primitive red cells very scanty, 0·6 per cent.; polymorphs, 35 per cent.; lymphocytes, 50·2 per cent.
Metamyelocyte (Neutrophilic)	17·4	13·0	12·6	4·4	3·5	3·0	0·0	
Polymorph (Neutrophilic)	16·4	30·4	24·4	35·6	0·5	1·0	0·0	
Myelocyte (Eosinophilic)	0·2	1·8	0·6	0·2	0·0	0·0	0·0	*18th and 21st* Areas of primitive cells appeared on 18th September but by 21st September they have disappeared and marrow appears acellular.
Metamyelocyte (Eosinophilic)	3·4	5·4	2·0	1·6	0·0	0·0	0·0	
Eosinophil (Eosinophilic)	0·0	0·0	0·6	4·6	0·0	0·0	0·0	
Basophil	0·4	0·2	0·4	0·0	0·0	0·0	0·0	
Lymphocytes	8·6	19·6	35·4	50·2	79·0	45·0	53·0	
Monocytes	3·2	2·8	3·6	0·0	0·0	0·0	1·0	
Plasma cells	0·2	0·2	0·0	0·0	1·0	2·0	0·0	
Megakaryocytes	0·0	0·2	0·0	0·2	1·0	0·0	0·0	
Reticulum cells	0·0	1·0	0·0	2·2	8·5	19·5	45·0	

*Normal Count.

47th day again disappeared (Table 7). When the calf was killed on the 49th day there were few myeloid cells in the marrow. Sections of the sternum showed erythroid hyperplasia, the cells being mainly intermediate and late normoblasts.

Peripheral blood. Platelets were reduced to 160,000 per c.mm. on 33rd day; white cells to 2,600 (2 per cent polymorphs) on 40th day. By the 47th day, 5 days after the bone marrow showed activity, 14 per cent metamyelocytes were observed in smears of peripheral blood.

The Effect of Vitamins of the B Group on the Bone-marrow of Calves Fed on Fresh Green Bracken (Experiment 3)

Calf 12 was given 8 kinds of group B vitamins intramuscularly. Calf 13 received 2 lb. grass daily in addition to its diet of bracken, and also received 750 mg. vitamin B^1 daily directly into the rumen.

The course of the disease and the bone marrow damage were not modified in any way by the administration of these vitamins. Calf 12 lived 31 days and Calf 13, 49 days after eating bracken.

The Effect of Transferring Rumen Liquor (Experiment 4)

To test the hypothesis that a poison may be formed in the rumen of cattle eating bracken, transfers of rumen liquor were carried out as described under experimental procedures. The liquor from a heifer on a bracken diet was transferred to the rumen of Calf 7 on a diet of concentrates, swedes and hay. The result of this experiment was that Calf 7 continued to gain weight at an average rate of 0·7 lb./day and no changes from normal were observed in the sternal bone marrow throughout the experimental period of 42 days. At the termination of the experiment, the heifer eating bracken was showing clinical signs of bracken poisoning.

While it is possible that insufficient toxic principle was transferred in the rumen liquor from the bracken-fed heifer, it may also be possible that the normal rumen microflora of the calf was capable of inactivating any such poison.

The Effect of Transferring Rumen Liquor (Experiment 5)

To test the hypothesis that normal rumen liquor might contain a nutrient essential for the integrity of the bone marrow of cattle fed on bracken, rumen liquor was transferred on separate occasions from a heifer fed normally to the rumens of a heifer and 3 calves fed on dried green bracken (1952 sample) and of 2 calves fed fresh green bracken (1954 sample).

The transfer of rumen liquor from the normal heifer to the bracken-fed animals had no effect on the outcome of the disease and all 6 animals ultimately died or were killed in extremis.

Bone marrow and peripheral blood. The bone marrows of these six animals were all damaged in a similar way to those of animals fed on bracken only or on bracken and grass. All showed the steadily diminishing cellularity which we had previously observed (Figs. 1-6).

TABLE 5

RESULTS OF FEEDING STEAMED BRACKEN TO CALVES

Calf No. and sex	*Days lived after eating bracken*	*Weight (lb.) during experiment*		*Average daily consumption of food (to appetite)*	*Remarks*
		Beginning	*End*		
32 ♀	30	194	158	3 lb. dried steamed bracken (1953 sample).	Steady loss of weight 1·2 lb./day. Bone marrow damaged after 19 days feeding on bracken. Died without signs of bracken poisoning. Heart rate 40 beats/min. Temp. 97°F.
48 ♂	30	190	183	20 lb. steamed fresh green bracken (1954 sample).	Weight maintained but rumen contents weighed 56 lb. at *post-mortem*. Bone marrow damaged after 10 days of experiment. Typical bracken poisoning. Pasteurella species isolated from blood when temperature rose to 107°F after 29 days feeding on bracken.
35 ♂	49	224	182	13½ lb. steamed fresh green bracken (1954 sample) + 1 lb. hay daily. After 39th day of experiment refused to eat bracken; offered on 44th day diet of linseed meal 2 parts: crushed oats 1 part but ate little (about 1½ lb./day).	Bone marrow damaged after 19 days of experiment. Bone marrow showed evidence of regeneration on the 42nd day. Developed abscesses in nasopharynx and tongue. Temp. 106°F. Blood culture negative. Killed 49th day. *Post-mortem:* Not typical bracken poisoning. No haemorrhages, but bone marrow showed myeloid aplasia and erythroid hyperplasia.

Occasionally in bone marrow smears large phagocytic reticulum cells became more noticeable in the last few days before death. The appearance of these cells four days before death was a prominent feature in the sternal marrow of the heifer used in this experiment (Figs. 9 and 10). We are unable to state definitely whether these cells represent a true hyperplasia of reticulum cells or whether they merely appear to be present in larger numbers because of the disappearance of other cells. However, hyperplasia of these phagocytic cells seems to be the more likely explanation, especially as the heifer developed a large extra-peritoneal abscess towards the end of the experiment.

A phenomenon worthy of note was observed in the heifer used in this experiment. A few days after transfer of normal rumen liquor had begun, an increase of primitive cells was noted in smear preparations from the marrow; 12 per cent of the marrow cells counted were primitive or early myeloid cell precursors and 45 per cent were metamyelocytes. Maturation of the metamyelocytes to neutrophile polymorphs took place during the next 6 days; this was reflected in an increase of white cells in the peripheral blood from 5,000 per c.mm. before transferring rumen liquor, to 11,600 (polymorphs 4,500) 10 days after the transfer had begun. During an interval of 14 days without rumen liquor transfers, the marrow again showed diminished cellularity. Transfers were recommenced and there was again a slight increase in marrow activity which was reflected by a rise in the numbers of polymorphs and platelets in the peripheral blood. However, despite continued transfer of an increased quantity of rumen liquor, the bone marrow continued to degenerate and the heifer died on the 110th day of experiment.

The phenomenon of reappearance of primitive cells late in the experiment also occurred in 3 calves of this experiment (Figs. 14-16 and 18-20). This apparent attempt at regeneration of the marrows of these calves was noted late in the experiment while the marrows were actually becoming less cellular as a whole and it is noteworthy that the appearance and maturation of these primitive cells was insufficient quantitatively to influence the peripheral blood counts.

The Provision of Extra Amino Acids or Extra Food via Abomasal Cannula (Experiment 6)

Some of the results of Experiments 1 to 5 described in this paper seemed to suggest that inanition may play a part in determining the bone marrow damage. In order to test this hypothesis, extra food or extra amino acids were given directly into the abomasum of bracken fed animals. These additions to the diet were given directly into the abomasum via a cannula in order to avoid prior breakdown by the rumen microflora.

Calf 40 given extra food gained 14 lb. during the first 21 days, but lost 15 lb. in the last 3 days of the experiment. Calf 33a given

TABLE 6

SERIAL DIFFERENTIAL COUNTS PER CENT OF STERNAL BONE MARROW

Calf 48 fed on steamed fresh green bracken**

Date		15th June*	25th June	1st July	7th July	12th July	Remarks on smears and histological sections
Haemocytoblast		0·8	0·4	0·4	0·2	0·0	*25th June:* Decrease in cellularity with relatively greater decrease in intermediate and late normoblasts.
Normoblast:							
Early		2·8	1·2	1·4	0·4	1·0	
Intermediate		24·8	4·0	8·4	1·6	1·0	
Late		28·6	3·2	6·8	1·0	1·0	*1st July:* Poorly cellular marrow.
Blasts		1·6	2·4	1·4	0·2	0·0	
Pro-myelocyte		1·2	2·4	0·8	0·2	0·5	*7th July:* Marrow cellularity steadily diminishing.
Myelocyte	Neutrophilic	0·8	2·0	2·4	0·4	0·0	*12th July:* Acellular marrow except for reticulum cells and endothelial cells of capillaries; capillaries dilated.
Metamyelocyte	Neutrophilic	11·6	24·0	21·4	6·4	0·0	
Polymorph	Neutrophilic	12·0	30·0	30·8	26·8	1·0	
Myelocyte	Eosinophilic	0·6	0·8	0·4	0·2	0·0	
Metamyelocyte	Eosinophilic	4·8	10·2	9·8	7·4	3·0	
Eosinophil	Eosinophilic	0·2	0·4	0·4	1·2	7·0	
Basophil		0·0	0·4	0·2	0·2	0·0	
Lymphocytes		5·2	13·4	12·0	47·2	68·5	
Monocytes		3·0	2·4	1·2	0·2	0·5	
Plasma cells		0·0	0·0	0·0	0·6	0·5	
Megakaryocytes		0·6	0·8	0·6	0·4	0·5	
Reticulum cells		1·4	2·0	1·6	5·4	15·5	

* Normal count.

** Serial differential counts and marrow changes were similar in a control calf which was fed the same sample of fresh green bracken unsteamed.

extra amino acids (casein hydrolysate) lost 11 lb. during the first 5 days of the experiment, but thereafter regained its original weight and maintained this weight till death. Both animals died in about 34 days from typical bracken poisoning. The extra food or amino acids had no effect on the course of the disease.

In contrast to calves fed on fresh green bracken alone, or on fresh green bracken plus hay or grass, the carcasses of Calves 33a and 40 contained subcutaneous, omental, perirenal and pericardial fat. The amount of fat in Calf 40 appeared to be within normal limits, but was slightly reduced in Calf 33a.

Bone marrow. Both calves had aplastic bone marrow at death. However, in Calf 33a (received casein hydrolysate), smear preparations from the biopsy 6 days before death showed a few primitive red cell precursors larger than normal (Fig. 8). These cells had a bigger, more reticular nucleus and had the appearance of pro-erythroblasts larger than normal rather than megaloblasts.

DISCUSSION

The type of bone-marrow damage described in these experiments can be classified as Aplastic type 1 (Whitby and Britton, 1950) where "primary division is at fault." In bracken poisoning the defect appears to be in the formation of blast cells; maturation from the blast cells seems to proceed at what may well be a normal rate, though whether cells undergoing maturation give rise to the normal number of daughter cells is not known. For example, examination of the bone marrow from a calf which had been eating bracken for some time, showed islands of blasts and promyelocytes where previous biopsies had shown these cells to be scanty. Eight to fourteen days later the bone marrow of the same calf showed that the predominant cells were metamyelocytes and polymorphs; blasts and promyelocytes had almost disappeared. The reappearance of these primitive cells during the course of continued feeding with bracken calls for comment.

The aplastic lesion we have studied in acute bracken poisoning is widespread throughout the sternal marrow; nevertheless as revealed by histological sections of bone marrow from calves killed on the 10th, 18th and 24th days of an experiment, or from calves in which the disease had run an acute course ending in death, small areas, cellularly more dense, persist. A similar phenomenon has been noted in marrow aplasia in man by January and Fowler (1940), who found small islets of well preserved marrow tissue in otherwise aplastic marrows. The presence of these small cellular areas in some of our experimental animals may help to account for the reappearance of blasts with sometimes subsequent though transient improvement in the peripheral blood picture.

The sun-dried bracken harvested in July, 1952 and fed to four animals induced changes similar to those observed in cattle or calves

TABLE 7

SERIAL DIFFERENTIAL COUNTS PER CENT OF STERNAL BONE MARROW

Calf 35 fed on steamed fresh, green bracken.

Date	*15th* July*	*26th July*	*6th Aug.*	*12th Aug.*	*23rd Aug.*	*26th Aug.*	*31st Aug.*	*2nd Sept.*	*Remarks on smears and histological sections*
Haemocytoblast	1·2	1·4	0·8	0·4	1·0	3·0	1·6	1·5	*26th July:* Relative decrease in erythroid precursors.
Normoblast:									
Early	4·4	2·4	1·6	1·2	1·5	7·0	5·2	4·5	
Intermediate	23·6	11·4	10·0	5·2	17·5	18·5	42·0	35·2	*6th–12th August:* Steady decrease in cellularity.
Late	27·0	12·2	8·2	4·6	8·5	14·5	27·0	42·5	
Blasts	1·2	2·6	1·6	1·0	0·5	4·0	6·4	0·5	
Pro-myelocyte	1·8	1·8	1·4	0·2	0·0	3·5	3·6	0·0	*23rd August:* Aplastic oedematous marrow except for reticulum cells and endothelium cells of capillaries; a few red cell precursors seen.
Myelocyte (Neutrophilic)	2·2	1·8	0·8	1·2	0·0	2·5	3·8	0·5	
Metamyelocyte (Neutrophilic)	18·2	24·0	22·2	15·4	1·0	3·0	0·0	2·5	
Polymorph (Neutrophilic)	13·4	22·6	32·4	27·4	0·0	0·0	0·0	1·0	
Myelocyte (Eosinophilic)	0·2	1·2	0·4	0·2	1·0	0·0	0·0	0·0	*26th August:* Increase in cellularity affecting primitive red cells and myeloid cells.
Metamyelocyte (Eosinophilic)	3·8	5·8	7·6	6·8	4·5	1·0	0·0	0·0	
Eosinophil	0·0	0·0	0·6	0·6	0·5	0·0	0·0	0·0	
Basophil	0·0	0·2	0·0	0·0	0·0	0·0	0·0	0·0	*31st August:* Blast cells increased to 6·4 per cent.
Lymphocytes	1·6	9·4	7·2	31·4	59·0	34·0	8·2	8·0	
Monocytes	0·2	1·6	2·0	2·4	0·0	0·0	0·0	0·0	*2nd Sept.:* Cellular marrow with red cell precursors predominating. Post-mortem sections showed areas of erythroid hyperplasia and many promegakaryocytes.
Plasma cells	0·0	0·0	0·0	0·2	3·5	1·0	0·6	1·5	
Megakaryocytes	0·0	0·4	0·8	0·0	0·0	0·0	0·6	0·0	
Reticulum cells	1·2	1·2	2·4	1·8	1·5	8·0	1·0	2·0	

*Normal count.

fed on fresh green bracken, i.e. bone marrow damage resulting in thrombocytopenia and leucopenia; this in turn was followed by widespread haemorrhages, accompanied by high temperature and bacteraemia. Calves fed on sun-dried bracken which was more mature and fibrous and was harvested in late August 1953 from a different area, did not develop this clinical picture. These calves steadily lost weight. In the later stages their temperatures were subnormal (96-98°F.). There was no bacteraemia, nor were there widespread haemorrhages. Except for an increase in red cells and haemoglobin and a moderate thrombocytopenia the peripheral blood values were unchanged. The bone marrow at death though not normal was still quite cellular in four out of six calves.

Moon and Pal (1949) made digestibility trials on 2 sheep and 3 bullocks with different samples of bracken. They found that fresh cut bracken in July had a Starch Equivalent of 46·4 but later, in October, digestibility dropped and S.E. was only 4·0. Comparison of the results of our experiments using different samples of dried bracken clearly indicates that the sample cut in late August, 1953 was not very nutritious and the calves eating this sample suffered some degree of inanition; also, the poisonous principle may have disappeared from the mature bracken. It would appear that inanition alone may be sufficient to account for the changes from normal seen in the sternal marrow of calves eating this bracken. It may well be that when bracken poisoning occurs in the field, the severity is enhanced by insufficient food.

During June to July 1954, fresh green bracken which had been "steamed" for one hour at 100°C induced bone marrow damage in Calf 48 at the same rate as the same sample of unsteamed bracken induced changes in a control calf. Both calves developed the typical haemorrhagic syndrome of bracken poisoning in 30 days. In Calf 35, fed during July to September, the bone marrow was damaged more slowly. Although this calf's temperature rose to 106°F. after 42 days on experiment it did not have haemorrhages or bacteraemia despite a platelet count of $0{\cdot}52 \times 10^5$ and a white cell count of $2{\cdot}0 \times 10^3$ (polymorphs 2·0 per cent). Towards the end of the experiment this animal went off its food and this was associated with the development of a large ulcer on the dorsum of its tongue and a large ulcerated mass of necrotic tissue in the posterior wall of its pharynx. It is thus demonstrated that an aplastic condition of the marrow such as occurs in bracken poisoning may occasionally lead to local sepsis of such severity as to interfere with normal food intake. It is a matter for further investigation rather than speculation whether the steaming of the later-cut bracken may have destroyed a factor responsible for damaging the capillaries or whether there is some other reason for the absence of massive haemorrhage.

From the evidence we present it seems that in fresh green bracken, particularly in bracken cut early in the season, the active principle which damages the bone marrow withstood heating to

100°C for 1 hour. It is therefore difficult to accept the conclusion of Evans, Evans and Hughes (1954) that steaming bracken renders it innocuous to cattle. However, it should be noted that the experiments of Evans, *et al.* differed in some respects from ours. For example, we steamed the active bracken while it was still fresh (Exp. 2, b and c). Evans, *et al.* dried and milled or chaffed their bracken before steaming. Further, in their 1949 experiment (their table no. 2, p.304) the sample of bracken which they steamed had apparently become inactive before steaming commenced, since they report that a control animal given the same sample of bracken unsteamed also survived the experiment. On the other hand, in their 1950 experiment, all three animals fed on steamed bracken survived, whereas 5 other animals fed the same bracken sample unsteamed died of bracken poisoning. Nevertheless, since they do not report any bone marrow studies on the animals fed on steamed bracken, it is difficult to assess the innocuous effect on peripheral blood observations alone.

Transferring rumen juice from a heifer fed on bracken to the rumen of a calf fed normally, did not induce bone-marrow changes in the calf during a period of six weeks. This experiment was based on the hypothesis that a poison may develop in the rumen of cattle fed on bracken. It is possible that the amount transferred was insufficient, but as no changes whatever were observed in the bone-marrow it may be that the normal rumen micro-organisms of the calf were capable of destroying any such poison.

The failure to influence the bone marrow picture in the experiments in which normal rumen juice was transferred to the rumen of animals fed on bracken, would seem to indicate that normal rumen juice did not contain a factor protective to the bone marrow of bracken fed animals. It may be argued of course that the quantities transferred were too small, or that a specific protective substance was inactivated in the rumen of bracken fed animals, but these alternative possibilities seem less likely than the absence of a specific protective factor in normal rumen juice.

It is unlikely that the transient bone marrow regeneration noted in Experiment 5 was due to rumen liquor transfer, because this phenomenon was also occasionally noted in calves on a diet of fresh green bracken only. We have previously described spontaneous improvement in peripheral blood in three heifers not given rumen juice (Naftalin and Cushnie, 1954 a). That the provision of extra food or casein hydrolysate did not prevent calves from dying of bracken poisoning, suggests that the bone marrow damage is not associated with the lack of an essential amino acid.

In conclusion, the evidence presented in this paper suggests that a poison is mainly responsible for the pathological changes seen in "bracken poisoning" in cattle. Further experimentation is necessary to determine the nature and mode of action of the toxic agent.

CONCLUSIONS

Bone marrow damage in calves fed on fresh green bracken was observed 10 days after the experiment began. The changes were a diminution in the degree of cellularity of the bone marrow and a relative (percentage) reduction in the intermediate and late normoblasts. The terminal picture was aplasia.

Regeneration, with the reappearance of blasts cells which subsequently matured, was observed during the course of the feeding on bracken; subsequently the regeneration was not maintained and the animals died of bracken poisoning.

Dried bracken fed to a heifer and to calves induced different changes in the bone marrow, depending on the sample of the bracken. A sample harvested and sun-dried in July 1952 induced typical bracken poisoning when fed in January to April, 1953; a more fibrous sample harvested and sun-dried in late August 1953 led to changes associated with inanition.

Heating fresh green bracken to 100°C (in steam for 1 hour) did not render the bracken innocuous. A calf fed a sample gathered and steamed in June to July 1954 died after 30 days from acute bracken poisoning; a calf fed a sample gathered in July to September 1954 developed severe bone marrow damage and sepsis with a temperature of 106°F.

The administration of group B vitamins parenterally, or vitamin B^1 directly into the rumen, did not alter the course of the bone marrow damage.

The provision of extra food to 2 calves via abomasal cannula did not prevent bracken poisoning but did improve the nutrition of the calves. In the calf receiving extra amino acids, proerythroblasts larger than normal appeared in the bone marrow on the 27th day of experiment.

Transfers of rumen liquor from a heifer fed on active bracken to the rumen of a calf fed normally did not induce any bone marrow damage in the calf. Transfer of normal rumen juice to the rumen of calves fed on bracken did not in any way affect the bone marrow damage.

The evidence presented suggests that a poison rather than a specific nutritional deficiency is the cause of the bone marrow damage.

REFERENCES

Evans, W. C., Evans, E. T. R., and Hughes, L. E. (1954). *Brit. vet. J.*, **110**, 295, 365, 426.

January, L. E., and Fowler, W. M., (1940). *Amer. J. clin. Path.*, **10**, 792.

Moon, F. E., and Pal, A. K. (1949). *J. agric. Sci.*, **39**, 296.

Naftalin, J. M., and Cushnie, G. H. (1954a). *J. comp. Path.*, **64**, 54; (1954b). *Ibid.*, 75.

Whitby, L. E. H., and Britton, C. J. C. (1950). *Disorders of the Blood* p. 416, 6th Edition, Churchill; London.

[*Received for publication, March 19th,* 1956]

Plate 1.

Fig. 1. Before the experiment began. Normal marrow. Note aggregates of red cell precursors.
Fig. 2. After 10 days of feeding on fresh green bracken. Decreased cellularity and disappearance of aggregates of red cell precursors.
Fig. 3. After 18 days. Further decrease in cellularity.
Fig. 4. After 24 days. Small areas of oedema of stroma.
Fig. 5. After 29 days. Oedema of stroma; small haemorrhages; capillaries dilated; aplastic marrow.
Fig. 6. Section of sternum of Calf 41 (Expt. 1b), fed on fresh green bracken and killed on 24th day of experiment. Aplastic marrow; capillaries and arterioles appear undamaged; capillaries dilated.
Figs. 1 to 5. Histological sections of flecks of sternal marrow obtained by biopsies from Calf 46 fed on fresh green bracken (Expt. 1b). H & E × 260.

Plate 2.

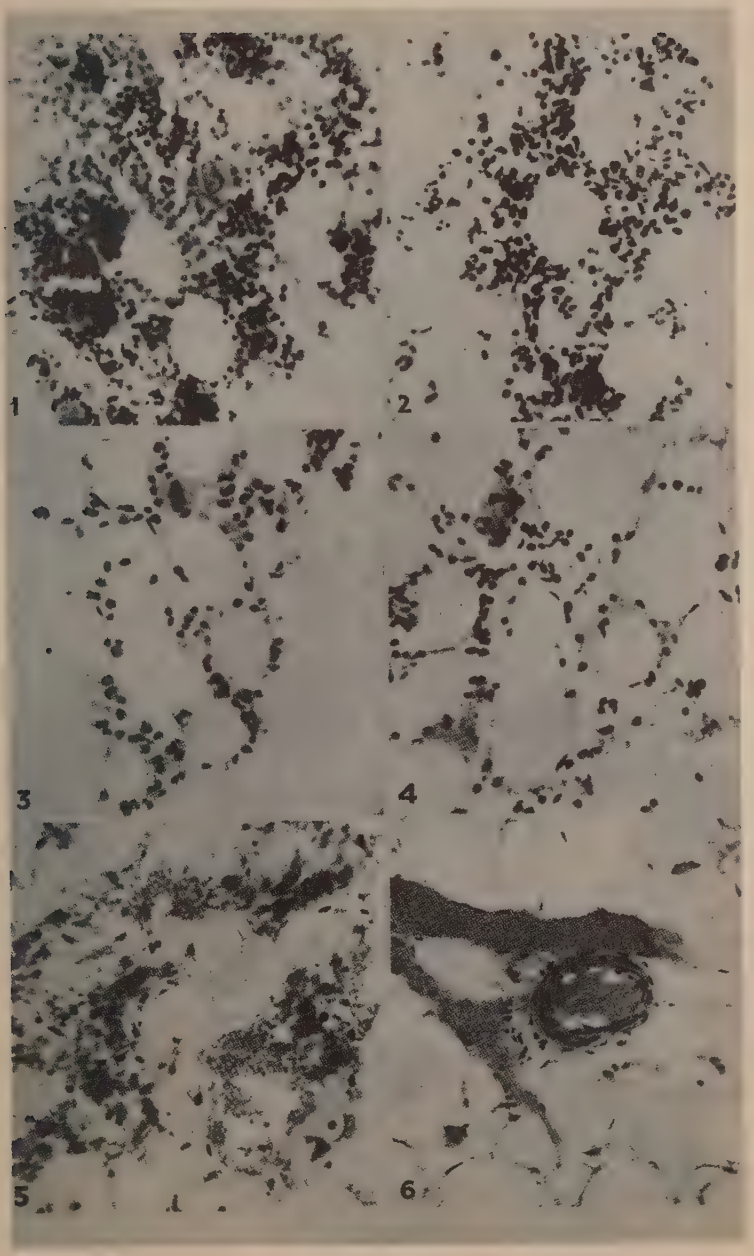
Fig. 7. Normal red cell precursors. From left to right. Intermediate normoblast; haemocytoblast—note pseudopodia and hyaloplasm (faintly stained ring around nucleus); early normoblast; late normoblast—Leishman's stain × 800.
Fig. 8. Two large proerythroblasts in marrow of Calf 33A (Expt. 6) on 27th day of experiment. Nucleus more reticular than normal. Leishman's stain × 800.
Figs. 9. and 10. Large phagocytic reticulum cells in marrow of Heifer 1 (Expt. 5) on 107th day of expt. Note ingested clumps of bacteria, and vacuoles. In Fig. 10 three cells are jammed together. Leishman's stain × 800.
Figs. 11. and 12. Pathological megakaryocytes Calf 46 (Expt. 1b) 17th day of experiment. The nuclei of these cells are fragmented. The cells are smaller than normal. Leishman's stain × 500.
Fig. 13. Normal megakaryocyte. Single lobulated nucleus; markedly granular cytoplasm. Leishman's stain × 600.

Plate 3.

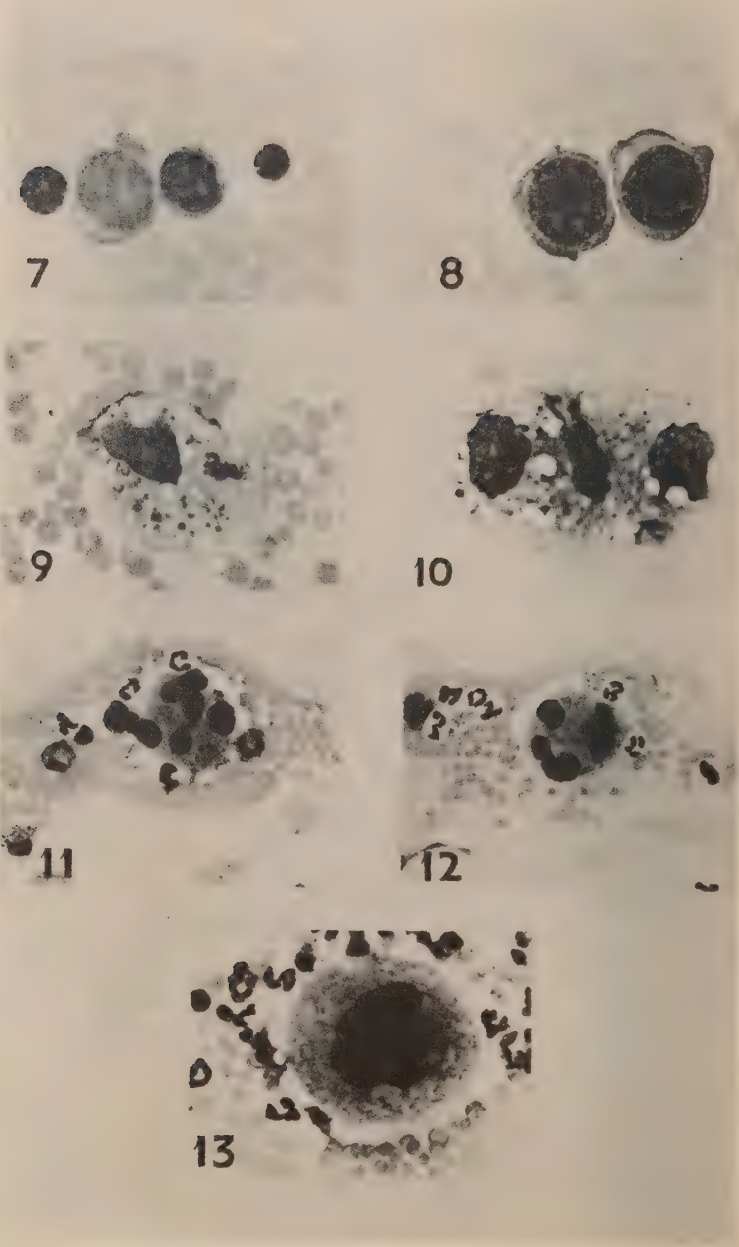
Fig. 14. Clumps of promyelocytes enmeshed in reticulum in smear preparation of Calf 8 (Expt. 5) on 39th day of experiment. In a previous smear (32nd day) blast cells and promyelocytes were absent.
Fig. 15. Lower centre. Cells mainly myelocytes in the "clumps;" upper left groups of intermediate and late normoblasts. 43rd day of experiment.
Fig. 16. Upper right; cells mainly myelocytes and polymorphs. Upper left; groups of intermediate and late normoblasts. 46th day of experiment.
Fig. 17. Section of sternum of Calf 26 fed on dried bracken (1953 sample) Expt. 1a killed on 27th day of experiment. Relatively acellular but not aplastic marrow; note dilated capillaries H & E × 240.
Figs. 14 to 16. Show apparent regeneration in a degenerating marrow. Bone marrow smear preparations of Calf 8 (Expt. 5). Photographs of small flecks of marrow. Leishman's stain × 240.

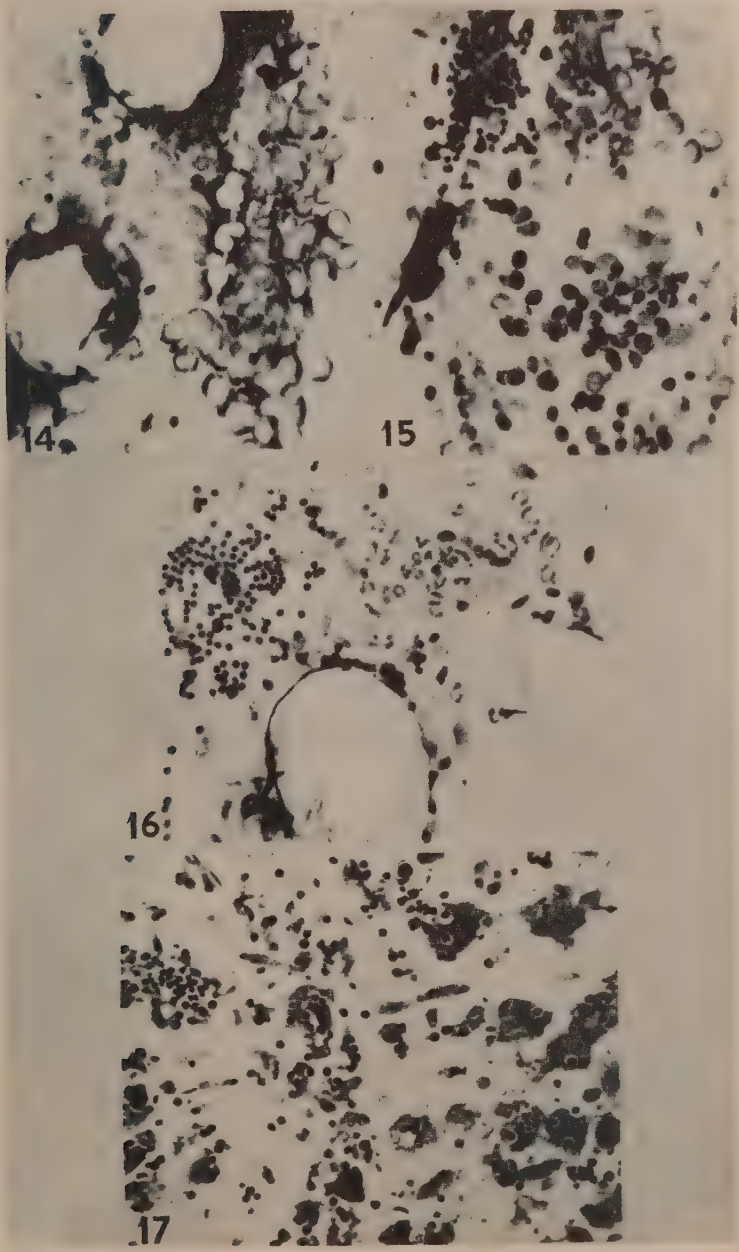
Plate 4.

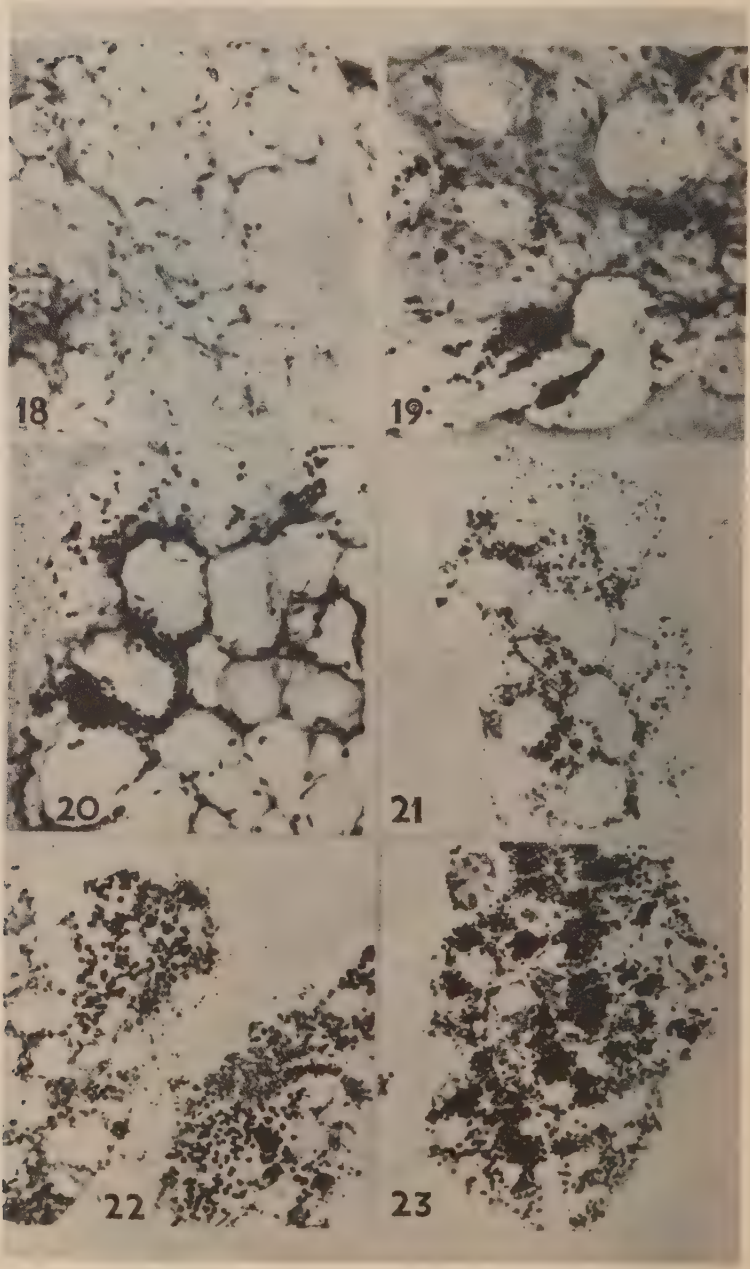
Fig. 18. Aplastic marrow on 36th day of experiment.
Fig. 19. Groups of early and intermediate normoblasts appear. 39th day of experiment.
Fig. 20. Groups of late normoblasts persist; oedema of stroma. 46th day of experiment.
Figs. 18 to 20. Show apparent regeneration in a degenerating marrow. Bone marrow smear preparations of Calf 9 (Expt. 5). Photographs of small flecks of marrow. Leishman's stain × 170.
Fig. 21. On 33rd day of experiment relatively acellular marrow.
Fig. 22. On 42nd day of experiment. Increase in cellularity principally of red cell precursors.
Fig. 23. On 49th day of experiment. Erythroid hyperplasia. Note sections of sternum post mortem showed widespread erythroid hyperplasia with many megakaryocytes but few myeloid cells. For details of feeding see text.
Figs. 21 to 23. Histological sections of flecks of sternal bone marrow obtained by biopsy from Calf 35 fed on steamed fresh green bracken (Expt. 2c) H & E × 170.



To face page 372


7
8
9
10
11
12
13


14
15
16
17


18
19
20
21
22
23

MORPHOLOGY OF THE ELEMENTARY BODIES AND CELL INCLUSIONS IN SWINE POX

By

F. Blakemore*

and

M. Abdussalam†

Department of Animal Pathology, Cambridge University

INTRODUCTION

The natural existence of two pox diseases in swine is now well known. One of these is caused by vaccinia virus and the other by a pox virus peculiar to piglets. Vaccinia is the severer of the two diseases and occurs in pigs of all ages. The other disease is considerably milder and is generally restricted to young pigs. It is not transmissible, with certainty, to any other animal species and may be regarded as the true pox of pigs (swine pox).

Blakemore and Glover (1937) reported the occurrence of swine pox in England and proved by transmission experiments that it was restricted to young pigs. Unlike some other workers who studied the disease in central Europe (Bendinger, 1934; Bendinger, Shilin and Zaitzew, 1935), America (Shope, 1940) and Japan (Terakado, 1953), these authors were unable to find any cell inclusions in sections of naturally infected skin. This was probably due to the advanced age of the lesions studied. In the present investigation cytoplasmic as well as intranuclear inclusions were found in the early lesions produced by a strain of the virus isolated from a natural outbreak in East Anglia. The opportunity was also taken to study the morphology of elementary bodies of the virus.

MATERIAL AND METHODS

Skin scrapings from a piglet bearing advanced scabby lesions were suspended in normal saline solution and inoculated by scarification on a three-month old piglet, an adult rabbit and a guinea-pig. In the piglet alone erythema developed on the fourth day, papules on the fifth, vesicles on the seventh and, subsequently, pustules followed by scab formation. By the 30th day the scabs had fallen off. The strain was then passed, in series, in three more piglets; the disease produced resembled that observed in the first piglet.

Pieces of the affected skin were biopsied on the seventh and 19th days after infection and fixed in Heidenhain—Susa fixative. Sections were stained with haematoxylin-eosin, iron-alum haematoxylin and with Weigert's stain.

Smears of papular, vesicular and vesiculo-pustular lesions were stained for elementary bodies. Relatively pure suspensions of these

*Deceased. †Present address; Lahore, Pakistan.

bodies in McIlvaine's buffer (M/250, pH.7·2) were obtained from the skin lesions by differential centrifugation as in vaccinia and other pox diseases. Such suspensions were also used for tinctorial studies and electron micrography.

RESULTS

Elementary Bodies

We were able to demonstrate numerous elementary bodies stained by Morosow's method in smears of scrapings of skin lesions taken before pustule formation. Better preparations were, however, obtained when the smears were stained by Paschen's method as modified by van Rooyen and Illingworth (1944) and Abdussalam (1948). In these preparations, the presence of thick exudate and particulate tissue debris did not overshadow the elementary bodies, as was often the case when Morosow's method was employed. For staining smears of washed elementary bodies, however, both methods were equally satisfactory. A fainter staining of elementary bodies was obtained with Victoria blue, methyl violet and Giemsa stains respectively.

In Paschen-stained smears, the elementary bodies appeared as small coccus-like particles disposed singly, in pairs, or small chains and, at places, in clusters. They measured 250 to 300 mμ in size. These measurements are considerably smaller than those of electron micrographs. With the ordinary microscope, elementary bodies of swine pox could not be distinguished morphologically from those of vaccinia which also occur in pigs in nature: nevertheless, this method can be usefully employed in studying clinical and experimental swine pox.

Electron Micrography

A relatively pure suspension of swine pox elementary bodies was obtained by a method of differential centrifugation similar to that used for isolating the elementary bodies of vaccinia and other animal pox viruses. Fairly rich suspensions were obtained when scrapings of lesions taken 5 to 12 days after inoculation were centrifuged; and some could still be isolated in scrapings of scab-covered lesions taken 19 days after infection.

Conventional micrographs of the elementary body showed it to be cuboidal to brick shaped with markedly rounded ends and edges. Internally, it showed one or two dense spots with higher electron absorbing power. One of these spots was usually in the centre. In the preparation studied, some small particulate matter was found scattered in the background or adsorbed to elementary bodies but it appeared to be of extraneous origin (Fig. 1). Electronic shadows of 24 elementary bodies were measured. They ranged in length from 208 mμ to 422 mμ with a mean length of 323 $\pm$13·9 mμ. The breadth varied between 164 mμ and 328 mμ with a mean of 239·9 $\pm$9·5 mμ. In gold shadowed preparations, the elementary bodies

were noticed to settle on the grid with about the same degree of collapse as did those of vaccinia and fowl pox.

No evidence of division of elementary bodies could be seen in any of the micrographs studied.

TABLE 1

DIMENSIONS OF ELECTRONIC IMAGES OF POX VIRUSES OF PIGS

Virus	*No. of elementary bodies measured*	*Arithmetical mean and standard deviation (mμ)*					
		Length		*Breadth*		*Axial ratio*	
		(*a*)	(*σ*)	(*b*)	(*σ*)	(*a*/*b*)	(*σ*)
Vaccina* ..	70	262	28·2	209	23·9	1·25	0·124
Swine pox ..	24	323	68·2	240	46·6	1·35	0·31

(All measurements in mμ)

*After Ruska and Kausche (1943)

Cytoplasmic Inclusions

In seven-day lesions, the basal epidermal cells of the affected area showed swelling of the cytoplasm. In the next one or two layers towards the surface, the cells exhibited reticulation of the cytoplasm with the appearance of spaces between the fine fibrils, particularly around the nucleus. In the next few rows of cells, the fibrils receded towards the cell membrane leaving a cavity enclosing the nucleus and some granulated material. This material collected to form one to several inclusion bodies in each cell (Figs. 2 and 3).

The size varied from a minute granule to a body slightly larger than the nucleus. Most of the bodies consisted of loosely packed groups of minute granules, while others were more compact though they also showed a granular structure. There was no enclosing membrane; hence, the shape varied from irregular grape-like clusters to round or ovoid masses.

When stained, the bodies were eosinophilic but in the deeper cells some basophilic spots were also seen.

Intranuclear Inclusions

At about the same level as that of the cytoplasmic inclusions, the nuclei of the affected cells showed round or ovoid inclusions which appeared as empty spaces, or poorly stained structures in haematoxylin-eosin stained sections. Their chemical nature could not be ascertained with certainty (Fig. 4). In all the affected nuclei the chromatin and the nucleolus were pushed to the periphery but were not uniformly distributed along the margin. In some nuclei the nucleolus degenerated.

Because of the intranuclear inclusion, which may be liquid or

semi-liquid, the nucleus showed no evidence of collapse in the otherwise degenerated cells of the epidermis till it was quite near the stratum corneum. In other pox diseases, in which reticular degeneration of the Malpighian layers of the epidermis takes place, the nucleus collapses earlier, i.e. in the deeper layers.

Neither cytoplasmic nor intranuclear inclusions were found in the 19-day old lesions covered over with scabs.

DISCUSSION

Morosow (1936) and Shope (1940) demonstrated the elementary bodies of swine pox in smears of early lesions stained by Morosow's silver deposition method. In the present study, this finding was confirmed but it was found that better results could be obtained with a modification of Paschen's method. Elementary bodies could be demonstrated in papular, vesicular and, to some extent, in vesiculopustular lesions. It is considered that this method is a useful diagnostic aid in experimental as well as clinical work.

Morphologically, the swine pox elementary body appears to be closely allied to the elementary bodies of the other pox viruses. It is, however, distinguishable from that of vaccinia, the other pox disease occurring in pigs, in being somewhat larger and longer.

Since Bendinger (1934) first described cytoplasmic inclusions in swine pox, they have been seen by other workers who studied the histology of skin lesions (Bendinger, *et al.*, 1935; Shope, 1940; Terakado, 1953). Some other careful observers, however, missed them completely (McNutt, Murray and Purwin, 1929; Blakemore and Glover, 1937). This may be attributable to the fact that the cytoplasmic inclusion in swine pox is unstable and observable only in incipient lesions. Similar instability is shown by cytoplasmic inclusions in contagious pustular dermatitis of sheep (Saliyi, 1939; Abdussalam, 1953).

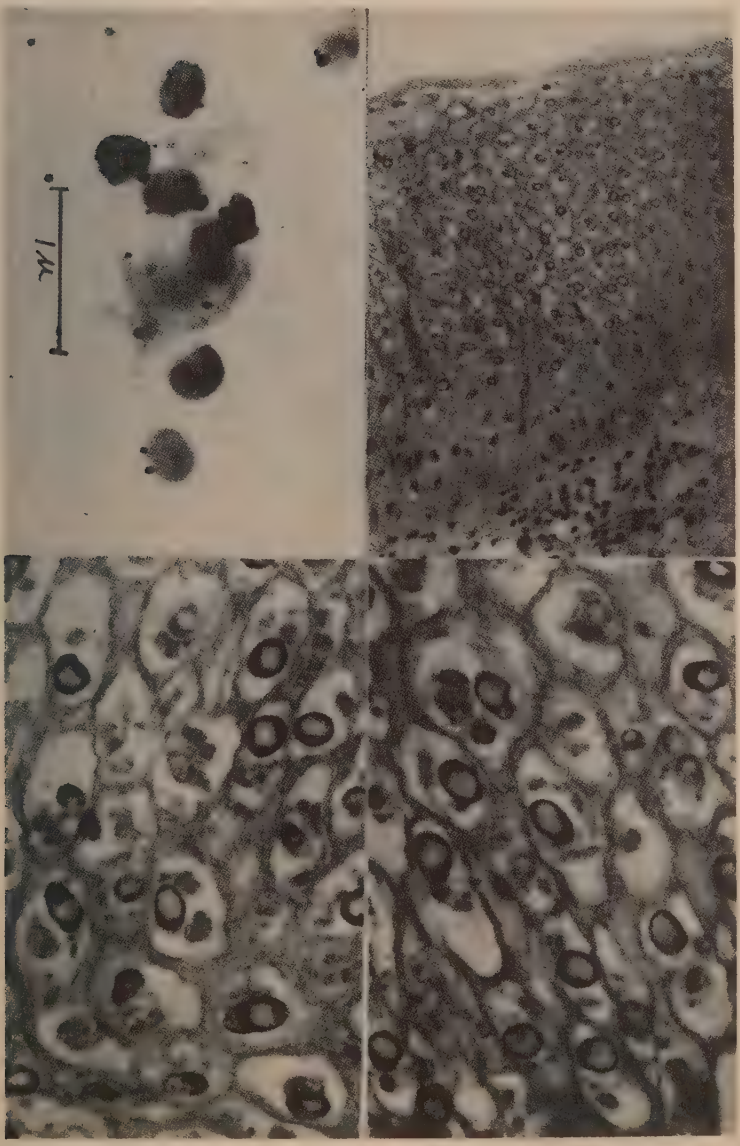
Intranuclear inclusions in swine pox do not appear to have been described before. Murray (1937) made a passing reference to their occurrence but does not appear to have studied them. Their most striking character seems to be chromophobia, i.e., inability to take up the stains used in this study.

CONCLUSIONS

Elementary bodies of the swine pox virus resemble those of other pox viruses. They are slightly larger and longer than those of vaccinia virus.

Cytoplasmic and intranuclear inclusions occur in epidermal cells in the early stages of the disease.

These observations provide fresh evidence that swine pox belongs to the pox group of diseases.



To face page 376

ACKNOWLEDGMENT

We are grateful to Mr. V. E. Cosslett for preparing the section on the electron micrography.

REFERENCES

Abdussalam, M. (1948). *Thesis,* Cambridge University; (1953). *Proc. 15th Int. vet. Congr. Stockholm,* **1**, 387.
Bendinger, G. (1934). *Arch. wiss. prakt. Tierheilk.*, **68**, 345.
Bendinger, G., Shilin, M. C., and Zaitzew, W. I. (1935). *Tierärztl. Rdsch.*, **41**, 269, 288.
Blakemore, F., and Glover, R. E. (1937). *Vet. Rec.*, **49**, 750.
McNutt, S. H., Murray, C., and Purwin, P. (1929). *J. Amer. vet. med. Ass.*, **74**, 752.
Morosow, M. A. (1936). *G. Batt. Immun.*, **17**, 324.
Murray, C. (1937). *J. Amer. vet. med. Ass.*, **90**, 326.
Rooyen, C. E. van, and Illingworth, R. S. (1944). *Brit. med. J., ii,* 526.
Ruska, H., and Kausche, G. A. (1943). *Zbl. Bakt., I,* **150**, 311.
Saliyi, G. (1939). *Allatorv. Lapok,* **62**, 329, 335.
Shope, R. E. (1940). *Arch. ges. Virusforsch.*, **1**, 457.
Terakado, Y. (1953). *Exp. Rep. Govt. exp. Sta. Anim. Hyg., Tokyo,* **26**, 91.

[*Received for publication, March 23rd,* 1956]

Fig. 1. Elementary bodies of swine pox virus. Electron micrograph.

Fig. 2. Transverse section of skin lesion of swine pox, seven days after inoculation. H.E. × 250.

Fig. 3. Part of section in Fig. 2, more highly enlarged to show cytoplasmic inclusions. × 950.

Fig. 4. Part of section in Fig. 2, more highly enlarged. Intranuclear inclusions are in focus. × 950.

FURTHER OBSERVATIONS ON THE CHEMOTHERAPY OF HISTOMONIASIS (BLACKHEAD) IN TURKEYS

By

C. Horton-Smith and P. L. Long

Houghton Poultry Research Station, Houghton, Huntingdon

INTRODUCTION

Furazolidone is effective in controlling infections with *Histomonas meleagridis* when fed to turkey poults before the initial intake of infective material occurs and it might also suppress infections when courses of treatment are started any time up to the tenth day after the initial infection with *Heterakis* ova (Horton-Smith and Long, 1956). The question of possible relapse after ceasing treatment was discussed by these authors who had reached much the same conclusions as those expressed by Joyner and Kendall (1955) in the account of their work with 2-amino-5-nitrothiazole. Some experiments were undertaken to determine the effect of varying the time and the duration of treatment on the course of histomoniasis. Both pre-infection and post-infection treatments were tested and the results obtained are recorded in this paper.

MATERIALS AND METHODS

Broad Breasted Bronze (B.B.B.) and British White (B.W.) turkey poults of mixed sexes and of different ages up to 11 weeks were used in the experiments. They were reared on wire floors and transferred to slatted floor units at 6 weeks of age. The starter and grower rations on which they were fed and the method of preparation of the medicated foods were similar to those already described by Horton-Smith and Long (1956). In two experiments the activity of furazolidone was compared with the known activity of 2-amino-5-nitrothiazole and 2-acetamido-5-nitrothiazole. For individual dosing the compounds were enclosed in gelatin capsules and administered per os. The infections were produced by administering approximately 500 *Heterakis* ova per os to each bird.

Medication, which was started either before or after initial infection with *Heterakis* ova, was continued for different lengths of time. The mortality from histomoniasis in treated groups during and after medication was compared with that in untreated groups. Examinations post-mortem were carried out on all surviving turkeys killed at the end of the experiments and, in one instance in the course of the test, on extracted samples of three individuals from each group, to determine the presence or absence of caecal and hepatic lesions.

RESULTS

The Effect of Continuous Medication starting before, and continuing after Infection

The medicated food was first offered two days before infecting fifty-eight 2-week-old B.B.B. poults with 500 *Heterakis* ova and medication was continued for periods of 15, 25, 40 and 60 days.

TABLE 1

FURAZOLIDONE (0.015 PER CENT.) BEFORE INFECTION AND FOR DIFFERENT LENGTHS OF TIME

Group	*Number of poults*	*Duration of treatment (in days)*	*Deaths from histomoniasis*																						*Total*
			Days after infection																						
			12	13	14	15	16	17	18	19	20	22	23	26	30	31	33	34	38	40	58	63	68	83	121	
1	14	15		S					1		1	1	1	1				1		1	K					7
2	14	25											S			3	1	1				1	K			6
3	14	40																	S					K		0
4	16	60																			S				K	0
5	20	—	2	3	1	4	2	2	1	2					K											17

K = survivors killed. S = treatment stopped.

TABLE 2

FURAZOLIDONE (0.015 PER CENT.) FOR 56 DAYS FROM THE DAY AFTER INFECTION

Group	*Number of poults*	*Deaths from histomoniasis*											*Total*
		Days after infection											
		15	16	17	18	19	25	57	60	69	103	105	
1	30			1 (a)				S	D	1	1(a)	K	3
2	30	9	11	2	3	3	K						28

(a) = caecal lesions only. D = died from causes other than histomoniasis.
K = survivors killed. S = treatment stopped.

Three groups (1, 2 and 3) were retained until the 45th day after the withdrawal of medication when all survivors were killed. The fourth group was not killed until the 63rd day after treatment had ended. A fifth group was infected in the same way as Groups 1, 2, 3 and 4 but received a non-medicated feed throughout. The survivors of this group were killed on the 30th day after infection. The results are given in Table 1. Two of the surviving three poults in Group 5 showed some evidence of caecal infection at autopsy but the livers of all were normal.

This experiment showed that treatment of 15 or 25 days' duration, even when started before initial infection with *Heterakis* ova, was insufficient to ensure a permanent suppression of *Histomonas* since fatal disease developed within 5 or 8 days of stopping medication. At the same time, however, about half of the poults survived in each case. Survivors were autopsied on the forty-fifth day (Groups 1 to 3) or sixty-third day (Group 4) after the end of treatment (Table 1). Caecal and hepatic lesions were found in one poult in Group 2 and thickening of the caecal walls in one in Group 3. No deaths occurred in Group 4, which was treated for sixty days, and the poults showed no lesions when they were killed sixty-three days after medication was stopped.

The Effect of Instituting Courses of Medication after Infection and for Different Durations

One group of 2-week-old B.B.B. poults received a medicated feed containing 0.015 per cent. w/w furazolidone from the day after infection for a total of 56 days and another was maintained on a non-medicated feed after infection. Of those on the medicated ration three were killed at 15 days and three at 30 days in order to determine the degree of infection present after these periods of treatment.

Two of the three poults killed at 15 days showed caecal lesions and *Histomonas* was recovered from the caeca. The livers were normal in all cases. None of those killed at 30 days showed caecal or hepatic lesions and no histomonads were demonstrated in the caeca. The results obtained in the main part of the experiment are shown in Table 2. Autopsies of the two surviving control poults killed 25 days after infection showed no lesions in either the caeca or livers.

The surviving poults in Group 1 were disposed of 105 days after infection. The caeca of one of the 26 birds contained some necrotic material. In view of the death occurring on the 103rd day, it would have been desirable, had circumstances permitted, to retain the survivors of Group 1 for a longer time. However, there was no evidence from the autopsies to suggest that disease would have developed in the remaining 25 poults since it seemed likely that the necrotic material observed in the caeca of the one poult would have resolved.

TABLE 3

FURAZOLIDONE (0.04 PER CENT) AT DIFFERENT TIMES AFTER INFECTION

Group	Number of poults	Medication (per cent) for 14 days	Delay of medication in days after infection	Deaths from histomoniasis: Days after infection 12	13	14	15	16	18	19	21	23	24	26	27	28	29	30	32	33	34	35	36	71	Total
1	20	0.04	5							S				8	2	3	1	3	1			1		K	19
2	20	0.04	10										S						4	1	2		1	K	8
3	18	0.04	12											S					1		2(a)			K	3
4	20	None	—	2	1	2	3	1	5	3	1	1				K									19

(a) = one bird with lesions in caeca only. K = survivor(s) killed. S = treatment stopped.

TABLE 4

FURAZOLIDONE (0.04 PER CENT.) FROM THE TENTH DAY OF INFECTION

Group	Number of poults	Medication (per cent) for 14 days	Delay of medication (in days after infection)	Deaths from histomoniasis: Days after infection 12	13	14	15	16	17	24	119	Total
1	20	0.04	10			2				S	K	2
2	20	None	—	2	1	4	5	6	1			19

S = treatment stopped. K. = survivors killed.

The Value of Furazolidone in the Treatment of Established Infection

In our previous paper (1956) a single experiment using 0.04 per cent. furazolidone in post-infection medication appeared to suppress the multiplication of histomonads completely and it was suggested tentatively, that this concentration of the compound might be used to control the disease. Two further experiments have now been done to test the efficacy of 0.04 per cent. furazolidone as a delayed treatment of *Heterakis* ova-induced histomoniasis.

In the first of these, three Groups (1, 2 and 3) of 2-week-old B.B.B. poults were given access to 0.04 per cent. w/w furazolidone from the 5th, 10th and 12th days after infection respectively. Medication was continued for fourteen days in all cases. Group 4 received non-medicated food. The mortality is shown in Table 3 from which it will be seen that there is a relation between the time treatment is started, its duration and its effect on the development of disease. There was a considerable reduction in mortality in Group 2 where treatment was delayed until the 10th day and this reduction was still more marked when the delay was extended to twelve days. The surviving poult from Group 4 which was killed and autopsied on the 28th day after receiving the *Heterakis* ova, showed no lesions. The surviving poults from Groups 1, 2 and 3 were killed 45 days after the treatment of Group 3 was completed and autopsy revealed a complete absence of caecal and hepatic lesions. A few *Heterakis* worms were recovered from Group 2 (5 worms) and Group 3 (6 worms).

In the second test with 0.04 per cent. w/w furazolidone, older turkeys of mixed breeds were used in order to ascertain the effect of treating older birds for fourteen days from the tenth day after infection. Two groups of mixed 8-week-old B.B.B. and B.W. turkey poults were used, Group 1 receiving medicated, and Group 2 non-medicated, food (Table 4). The surviving poults were killed 95 days after treatment had ceased, or 119 days after receiving the infective doses of *Heterakis* ova. Autopsy revealed normal caeca and slight scarring of the livers of two turkeys from Group 1. The sole survivor from Group 2 showed normal caeca and liver.

The results of the two tests indicated that although 0.04 per cent. furazolidone might afford substantial protection when administration was delayed until the tenth day and was continued for a further fourteen days, a total suppression of histomonad multiplication did not always occur.

Comparative Values of Different Drugs in Delayed Treatment

Sixty 2-week-old B.B.B. and B.W. turkey poults were infected and distributed among four groups of fifteen poults each. On the tenth day after infection and for fourteen days in all, Group 1 received 0.04 per cent. furazolidone, Group 2 0.1 per cent. 2-amino-5-nitrothiazole and Group 3 0.1 per cent. 2-acetamido-5-nitrothiazole, in the food. Group 4 received no medication. The results

TABLE 5

FURAZOLIDONE, 2-AMINO-5-NITROTHIAZOLE AND 2-ACETAMIDO-5-NITROTHIAZOLE FROM THE TENTH DAY OF INFECTION

Group	Number of poults	Medication in feed for 14 days	Deaths from histomoniasis: Number of days after receiving 500 ova											Total deaths	
			14	16	17	18	24	34	38	46	55	102	130	Histomoniasis	All causes
1	15	0.04% furazolidone					S	1(a)	2		1(a)			4	4
2	15	0.1% 2-amino-5-nitrothiazole					S							0	0
3	15	0.1% 2-acetamido-5-nitrothiazole					S			D		D		0	2
4	15	None	1	10	1	2								14	14

(a) = caecal lesions only. D = death from causes other than histomoniasis. S = treatment stopped.

TABLE 6

TREATMENT WITH FURAZOLIDONE ADMINISTERED TO INDIVIDUAL TURKEYS AFTER INFECTION

Group	Treatment with furazolidone	No. of poults in group	Total furazolidone administered in mg.	Mg. of furazolidone administered per kg. body weight	Total mortality	Findings at autopsy of survivors killed 49 days after infection: Lesions	
						Caeca	Liver
1	50 mg. on 3 successive days starting at time of symptoms in any poult of any group.	5	150	80	0	1 N	0
2	50 mg. on 3 successive days starting at time of death of first poult of any group.	5	150	80	1	0	0*
3	250 mg. given as single dose at time of death of first poult of any group.	5	250	146	2	0	0*
4	No treatment	5	—	—	5	—	—

N = small amount of necrotic material present. * = slight scars in 2 poults (group 2) and 1 poult (group 3).

over a period of 130 days are shown in Table 5. All three compounds gave good protection but furazolidone was less effective than the others. The surviving poults from all groups were retained and, at the time of writing, are over six months of age and in excellent condition.

The Effect of Medicating Individual Diseased Poults

Occasionally, dosing of individual poults may be desirable and a small scale experiment was done to test the effect of furazolidone on advanced infections when administered in three daily doses or as a single one. Four groups of infected 11-week-old B.B.B. turkeys were maintained together in one wire-floored pen. Group 1 was treated with three daily doses of 50 mg. furazolidone, the first of which was given to each poult at the first appearance of sulphur-coloured droppings in the pen, regardless of their origin. Group 2 was similarly dosed but here the treatment was started as soon as possible after the first death had occurred in any of the groups. The poults in Group 3 were treated with a single dose of 250 mg. at the same time as those of Group 2. Those in Group 4 were untreated. The results of this experiment are summarised in Table 6. Deaths occurred in the treated groups 2 and 3 between the 14th and 21st days of infection. All deaths in the control group (4) occurred between the 14th and 16th days of infection. At examination post-mortem the presence of necrotic material was noted in the caeca of one of the poults (Group 1) and scar formation in the livers of 2 survivors of Group 2 and one survivor of Group 3. These lesions suggested the residua of old rather than of active infections.

Comparison of Furazolidone and 2-amino-5-nitrothiazole

Although few turkeys were used in the previous experiment the results obtained seemed to justify a comparison of furazolidone with 2-amino-5-nitrothiazole administered in single doses over three consecutive days, the first dose being given 12 days after infection with *Heterakis* ova. For this purpose a batch of six-week-old mixed B.B.B. and B.W. turkeys was subdivided into three groups: six birds received 35 mg. furazolidone each on three consecutive days starting 12 days after the administration of *Heterakis* ova; six others were similarly dosed with the same quantity of 2-amino-5-nitrothiazole; seven birds were infected with the same number of *Heterakis* ova but were given no medication. The mortality occurring in the several groups is summarised in Table 7. All surviving turkeys were killed 55 days after receiving the last doses of furazolidone and 2-amino-5-nitrothiazole i.e., 69 days after infecting them with *Heterakis* ova. No caecal or liver lesions were found on examination post-mortem. The two compounds gave much the same protection and no relapses occurred in either group after the twentieth day following infection with *Heterakis*.

TABLE 7

TREATMENT WITH FURAZOLIDONE AND 2-AMINO-5-NITROTHIAZOLE ADMINISTERED TO INDIVIDUAL TURKEYS AFTER INFECTION

Group	*Number of poults*	*Treatment per poult per day for 3 days from the twelfth day of infection*	*Total drug administered (in mg.)*	*Dosage equivalent (in mg./kg.)*	*Deaths from histomoniasis*							*Total*
					Days after infection							
					14	15	16	17	19	20	69	
1	6	35 mg. furazolidone	105	170	S	+				+(a)	K	2
2	6	35 mg. 2-amino-5-nitrothiazole	105	170	S				+	+(a)	K	2
3	7	No treatment	—	—	+		++	++	+(+)			7

(a) = lesions in caeca only. K = survivors killed. S = treatment stopped. (+) = sacrificed *in extremis*.

DISCUSSION

The relative merits of medication in the prevention and treatment of histomoniasis have been discussed by various investigators. On the whole, current opinion favours prevention as being the only really effective means of dealing with this disease under field conditions. Little or no immunity to the disease apparently develops and it appears that a clinical infection, possibly with deaths, may arise from any sort of exposure to the causative organism. In contrast to some forms of coccidiosis in turkeys, epidemics of blackhead may develop slowly so that treatment of a small number of clinically affected turkeys within a flock affords no guarantee of control of the disease in the flock as a whole. Although short-term treatments may produce improvement in the condition of clinically affected turkeys, relapses may occur owing to the continued multiplication of the protozoan after treatment is withdrawn. The relation existing between relapse and the time that medication is initiated and its duration has been considered by Joyner and Kendall (1955). The results obtained in the present paper suggest that the duration of treatment is of importance in preventing relapse. When a group of turkeys was treated with 0.015 per cent. w/w furazolidone in the feed from the day after infection with *Heterakis* ova, and for a further 56 days, one poult died during treatment on the 17th day after receiving the infective dose. This death was followed by two deaths, one occurring 12 days and the other 46 days after treatment had ended. It seems unlikely that the 24 hour delay of the start of treatment after infection is necessarily responsible for these results. However, examinations post-mortem of poults killed 105 days after initial infection revealed no evidence of disease although one poult had died of the disease on the 103rd day. The tentative view expressed in our previous paper (1956) that permanent suppression of established *Histomonas* infections could be obtained by a medicated feed containing 0.04 per cent. w/w furazolidone is not confirmed by the results obtained in the present investigation. However, a marked control of 10 and 12 day old infections was obtained by treating poults for 14 days. It is interesting to note that the group which was treated for 14 days from the 5th day of infection received no benefit from medication. Furazolidone, at 0.04 per cent. w/w in the feed, may be of value in short-term treatment of established infections although relapses may occur if such treatment is not followed up by a course of treatment at the lower concentration of 0.015 per cent. The effects of delayed treatment, starting on the tenth day of infection and continuing for 14 days, were compared with those of 0.1 per cent. w/w 2-amino-5-nitrothiazole and 0.1 per cent. w/w 2-acetamido-5-nitrothiazole all incorporated in the feed. The results showed furazolidone to be inferior to the other two compounds. Although deaths from histomoniasis occurred on the 10th, 14th, and 31st days after the termination of treatment, some pro-

tection was afforded since only four of the fifteen poults in the furazolidone-treated group succumbed to the disease. The survivors of this group, as well as those treated with the other two compounds are now over six months of age and are in excellent condition. The results obtained in a small-scale experiment suggest that a three day course of single doses given to diseased turkeys might be of value in suppressing histomoniasis when applied at the onset of symptoms in any bird within a group. However, in comparing furazolidone with 2-amino-5-nitrothiazole, when used in daily doses of 35 mg. for three consecutive days, i.e. at the equivalent dosage of 170 mg./kg. body weight, which is rather less than half the dosage used in a previous experiment and beginning the course of treatment on the twelfth day of the infection, both compounds failed to give complete protection but no further deaths occurred after the twentieth day of infection, i.e. six days after the end of treatment. Examinations post-mortem revealed neither caecal nor liver lesions in any of the turkeys.

The experimental data provide further evidence of the value of furazolidone in the prevention of histomoniasis and indicate that the occurrence of relapse depends, to a considerable extent, on the length of treatment and the time that treatment is started relative to initial infection.

Cooper and Skulski (1956) have observed histological changes affecting spermatogenesis in the testes of S.C.W.L. chickens treated continuously with furazolidone from day old to maturity and have also observed that those changes may persist for 12 weeks after the withdrawal of treatment. Changes were also noted in the testes of immature and mature chickens which had received high concentrations of the drug for short periods. The extent to which the drug affects fertility after treatment has been withdrawn has not yet been determined. More recently, Cooper (1956) has shown that continuous feeding of furazolidone at concentrations of 0.01 to 0.02 per cent. to S.C.W.L. pullets during a period of 14 to 26 weeks of age had no significant effect on body weight, food consumption, date of first egg, egg-production, fertility and hatchability. The effects of treatment on the male gonads of fowls, as described by Cooper and Skulski, are sufficiently serious to warrant similar investigations with turkeys, and in the meantime, it is probably desirable to exercise caution in treating breeding stock although Moore, Chamberlain and Carter (1954) found no reduction in the fertility of furazolidone-treated turkeys.

CONCLUSIONS

Turkey poults receiving 0.015 per cent. w/w furazolidone in the food two days before infection with *Heterakis* ova were maintained on treatment for periods of 15, 25, 40 and 60 days. No deaths from histomoniasis occurred during the periods of treatment but relapses

occurred in the relevant groups after the withdrawal of the 15 and 25 day courses of treatment.

Some relapses occurred in turkey poults which received a 56 day course of treatment with 0.015 per cent. w/w furazolidone in the food starting from the day after infection with *Heterakis* ova. One poult died during medication. Relapses occurred on the 12th and 46th day after the end of medication.

Turkey poults receiving a 14 day treatment with 0.04 per cent. furazolidone from the 10th and 12th days of infection were protected during the period of medication, but some relapses occurred when medication was withdrawn. The number of relapses was few in the case of the group treated from the 12th day of infection.

Substantial protection from relapse was afforded by 0.04 per cent. w/w furazolidone in the feed when offered to poults for 14 days from the 10th day of infection, but the protection given was inferior to that obtained with 0.1 per cent. w/w 2-amino-5-nitrothiazole or 2-acetamido-5-nitrothiazole fed under similar circumstances of time and duration.

A series of high doses of furazolidone and 2-amino-5-nitrothiazole administered directly to individual turkeys with well established infections may prevent or reduce the number of deaths from histomoniasis.

The applications and limitations of treatment with furazolidone are discussed.

ACKNOWLEDGMENTS

We would like to extend our thanks to Dr. R. F. Gordon for his encouragement and to Miss M. Ward and Miss A. Brown for technical assistance. We are much indebted to Messrs A. J. White and Messrs. May & Baker for supplying the chemical compounds used in this work.

REFERENCES

Cooper, D. M. (1956). *J. comp. Path.*, **66**, 312.
Cooper, D. M., and Skulski, G. (1956). *Ibid.*, **66**, 299.
Horton-Smith, C., and Long, P. L. (1956). *Ibid.*, **66**, 22.
Joyner, L. P., and Kendall, S. B. (1955). *Vet. Rec.*, **67**, 180.
Moore, E. N., Chamberlain, V. D., and Carter, R. D. (1954). *Poult. Sci.* **33**, 1072. (Abst.)

[*Received for publication, April 4th*, 1956]

PERIODICAL